ISOLATION OF A SOMATOMEDIN BINDING PROTEIN FROM HUMAN PRETERM AMNIOTIC FLUID

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PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR IN DE
GENEESKUNDE
AAN DE ERASMUS UNIVERSITEIT ROTTERDAM
OP GEZAG VAN DE RECTOR MAGNIFICUS
PROF. DR. J. SPERNA WEILAND
EN VOLGENS BESLUIT VAN HET COLLEGE VAN DEKANEN.
DE OPENBARE VERDEDIGING ZAL PLAATSVINDEN OP
VRIJDAG 14 OKTOBER DES NAMIDDAGS
TE 3.45 UUR

DOOR

STENVERT LEONARD SEBASTIAAN DROP

geboren te Zeist

1983

Offsetdrukkerij Kanters B.V.,
Alblasserdam

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The first part of this study was performed in the Polypeptide Laboratory (heads: drs. B.I. Posner and H.J. Guyda), McGill University, Montreal, Canada, in 1977 and 1978.
The author received a research fellowship from the Montreal Children's Hospital Research Institute.
The study was continued in the Research Laboratory of the Sophia Children's Hospital, Rotterdam, and financially supported by the Sophia Foundation For Medical Research and by the Nordisk Grant For The Study Of Growth.

In memory of my father.
To my mother,
Henriette,
Alexander.

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PREFACE

Normal growth is the result of a very complex interaction between nutritional and hormonal factors and the responsiveness of the target organs. This holds equally true for fetal and postnatal growth.

Present knowledge about the hormonal control of fetal growth is briefly reviewed in chapter I. There are several indications, among which "experiments of nature", suggesting that hormones do play an important growth promoting role during fetal life. The presence of growth promoting factors in the fetus has long since been recognized, and many attempts have been made to isolate the active fetal factors.

One of the hormones involved in the regulation of postnatal growth is pituitary growth hormone (GH) or somatotropin. Although the hypothalamo-pituitary system rapidly develops notably early in fetal life and acromegalic values of GH are found in fetal serum, the role of GH during fetal growth is uncertain. In recent years, considerable attention has been focused on somatomedin (SM) or insulin-like growth factors (IGF), i.e., GH dependent, small molecular weight peptides with insulin-like activity, mediating the growth promoting actions of GH. It remains puzzling why during the pre- and perinatal period SM levels in serum or amniotic fluid, measured by various assay techniques, are unvariably very low. The presence, therefore, of a specific embryonic SM has been postulated.

SM circulates in plasma bound to macromolecular weight carrier or binding proteins. At least two forms (150-200,000 and 40-50,000 MW) in postnatal plasma have been recognized. The precise function of these binding proteins is unclear.

In addition to being carrier proteins, they may play an important role in the regulation of the biological effects of circulating SM.

This thesis study was undertaken in order to investigate the nature and biological behavior of a somatomedin binding protein, identified in preterm amniotic fluid.

In chapter II, the partial purification and characterization of a binding protein for SM (IGF-II) in human preterm amniotic fluid (AF) are described. This amniotic fluid binding protein (AFBP) inhibits the insulin- and SM-like effects of IGF-II in various in vitro bioassays. It proved necessary to further purify this binding protein in order to perform not only additional characterization studies, but above all in order to raise a specific antibody, allowing detection and quantitation in various body fluids.

In chapter III, an improved procedure is presented for the isolation of AFBP from preterm amniotic fluid, with high speed gel filtration chromatography (HSGFC) and disc-gel polyacrylamide gel electrophoresis as the final steps. In appendix 1, chapter III, the methodology of HSGFC, a recent advancement in protein purification, is given.

As is outlined in chapter IV, an antibody against AFBP was raised in rabbits and the antiserum cleared of contaminating anti-albumin antibodies. Subsequently, a specific radioimmunoassay (RIA) was developed. AFBP values measured by RIA in preterm AF (16-20 weeks of gestation) were twice as high as in term AF. Following Sephadex G-200 chromatography of serum at pH 2.2 and pH 7.4, AFBP-RIA activity was identified only in fractions at $K_{av} \pm 0.6$, identical to the elution volume of labeled AFBP.

In human fetal (20-22 weeks of gestation) and term cord serum, markedly elevated serum levels of AFBP were found

(chapter V). These contrasted with low IGF-I (SM-C) levels (measured in the same samples by RIA) and with decreased AFBP, but normal IGF-I/SM-C levels in serum of adults. In serum of children with GH deficiency, AFBP values were higher than in age-matched controls; they declined following GH therapy. In these patients, SM values were below normal for age, generally showing an increase during GH treatment. AFBP shows a striking similarity to an unsaturated SM binding protein, as described by Hintz (1981).

Speculations regarding the regulatory mechanisms for AFBP and its physiological importance are discussed in chapter VI.

The AFBP radioimmunoassay will facilitate a further elucidation of the physiological importance of AFBP, which may contribute to the understanding of SM action in pre- and postnatal growth.

CHAPTER I

HORMONAL REGULATION OF FETAL GROWTH

I.1. Introduction

Normal fetal growth depends upon a complex interaction between nutritional and hormonal factors and the responsiveness of the target organs. These factors lie at the root of many well-known maternal, placental and fetal causes of intrauterine growth retardation.

Several "experiments of nature" in humans suggest that hormones play an important growth promoting role during fetal life, but not necessarily in the same way postnatally (Cheek et al., 1977). Some of the hormones indispensable for postnatal growth, such as thyroid hormone and growth hormone, do not appear to be so prenatally. For instance, infants with complete thyroid hormone deficiency or with proven GH deficiency are of normal length at birth (Letarte et al., 1980; Cheek et al., 1977). Hyperinsulinemia or increased sensitivity to insulin results in overgrowth. A well-known example is the infant of a diabetic mother (IDM). Poor treatment of the insulin-dependent diabetic mother results in overgrowth of almost every tissue except the brain. These abnormalities disappear following good control of the maternal diabetes. Frequently, the infant producing an excess of insulin due to a tumor or nesidioblastosis, is clinically indistinguishable from the IDM. Increased numbers of insulin receptors in erythrocytes have been found in patients with Beckwith-Wiedemann-Syndrome (exophthalmus, macroglossia and gigantism), suggesting increased end-organ sensitivity to insulin resulting in overgrowth (Herzberg et al., 1979). Fetal overgrowth has beer experimental

ly generated in the fetal rat and monkey by direct injection or chronic infusion of insulin (McKormick et al., 1979; Susa et al., 1979). In contrast, fetal undergrowth has been observed in infants with hypo-insulinemia or decreased sensitivity to insulin, as found in pancreatic agenesis or end-organ receptor defects (leprochaunism) (D'Ercole et al., 1977; Taylor et al., 1982; Kaplowitz et al., 1982[a]). Although anencephalic infants are of normal length at birth, neither GH nor any pituitary tissue is detectable after careful postmortem examination (Cheek et al., 1977). From experiments monitoring the growth of the decapitated fetal rat, it has been concluded that the pituitary gland only has a minor influence on fetal somatic growth (Liggins, 1974). Of specific interest, however, is the important observation of the "experiment of nature" in anencephalics by de Gasparo and Hoët (1970): in anencephalics born to healthy mothers, the percentage of endocrine tissue in the pancreas, the number of beta-cells and the insulin cord values have been found to be the same as in normal infants. In anencephalics born to gestational diabetic mothers, pancreatic endocrine tissue, the number of beta-cells and cord insulin levels remained unchanged in the absence of a hypothalamus-hypophysis. Yet, in the presence of an intact hypothalamus-hypophysis in the above situation, the pancreatic endocrine tissue was enlarged, the cord insulin concentration high and birth weight and length were increased to the same degree as in the "normal" child of a diabetic mother. This "experiment of nature" suggests that the hypothalamic-pituitary axis acts through the endocrine pancreas to modify fetal growth.

Before discussing at length the intriguing recent development in research concerning the function in utero of somatomedin (SM) or insulin-like growth factor (IGF), i.e., peptide hormones postnatally mediating the growth promoting

actions of pituitary growth hormone, it seems fitting to first remark on some aspects of:

- the early development of the hypothalamo-pituitary system;
- the ontogeny of fetal GH;
- the somatomedin concept.

I.2. The development of the fetal hypothalamo-pituitary system

In contrast to previous understanding that the human pituitary gland develops from two distinct areas of the brain - ectodermal and neuroectodermal in origin -, recent evidence from anatomical studies in avian embryos suggests the Rathke's pouch to arise from the most caudal extension of the ventral neural ridge. Consequently, both the hypothalamus and the adenohypophysis can be regarded as embryonic derivates from a common neuroectodermal anlage (Takor et al., 1975). In vertebrates, most cells producing hormonal peptides belong to a family of cells known as the APUD series (amine content and/or precursor uptake and decarboxylation). These cells are all thought to be of neuroectodermal origin. This means that the hypothalamo-pituitary axis is not to be considered an interaction between classical neurons and endocrine cells, but a communication between two units of a complex peptidergic nervous system (Pearse et al., 1979).

The hypothalamus is the first of the forebrain regions to differentiate. It is recognizable at 22 days of fetal age. Between 6-12 weeks of gestation, a rapid differentiation occurs of various nuclei (supraoptic, paraventricular, dorsomedial, arcuate and mamillary). Neurotransmittors, such as dopamine, serotinin, norepinephrine and hypothalamic hormones (releasing factors), become detectable (Goodyer, 1981). The adenohypophysis develops from an outpocketing of the roof of the pharyngeal region

closely attached to the floor of the diencephalon (Rathke's pouch). At about 5 weeks of fetal age, a vascular network appears in the connective tissue immediately surrounding Rathke's pouch. Epithelial cells divide rapidly, forming small follicular structures. Neuroblasts begin to differen-

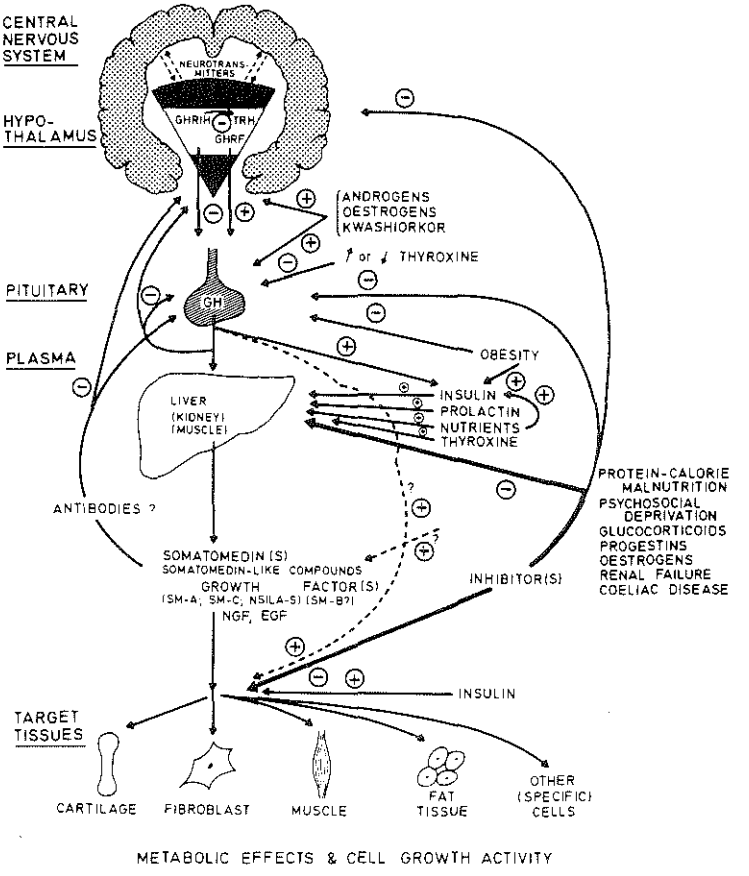

Fig. I.1.
Schematic diagram of the hypothalamo-hypophyseal unit, its hypothetical pathways and mechanisms involved in the secretion of growth hormone. (From: Vanderschuren-Lodewijckx, 1981; reprinted with permission of author and publisher.)

tiate in the overlying hypothalamus and a primitive neurohypophysis is formed. At 3 months of gestation, an anterior, intermediate and posterior hypophysis can be recognized. All pituitary peptides are demonstrable in specific granulated cells through immunohistochemical techniques (Goodyer, 1981). In the meantime, the vascular supply to the hypothalamic and pituitary region is developing into a complex portal system. Thus, the hypothalamus and pituitary are likely to interact at a very early stage of gestation. Maternal pituitary hormones do not cross the placenta, so the fetus is dependent on the secretions of its own pituitary, supplemented with various analogous hormones produced by the placenta. However, pharmacological doses of TRH given to parturient women have been shown to increase TSH and GH in cord blood (Roti et al., 1981, 1982).

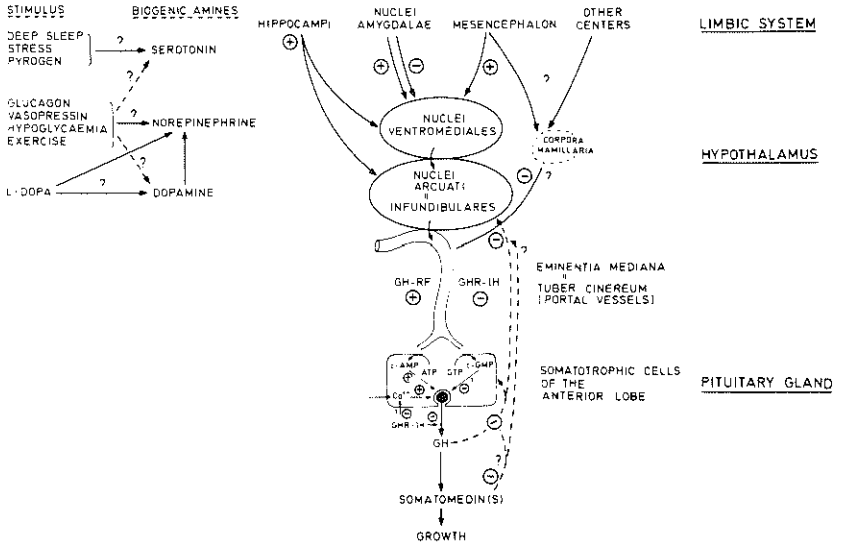

Fig. I.2.
Schematic diagram of the hypothalamo-hypophyseal unit and of hormonal interactions involved in the secretion of growth hormone (GH), production of somatomedin and somatic growth. (From: Vanderschuren-Lodewijckx, 1981; reprinted with permission of author and publisher.)

I.3. The ontogeny and regulation of fetal growth hormone

In postnatal life, GH is regulated by a dual mechanism consisting of the hypothalamic growth hormone releasing factor (GRF), the existence of which has been demonstrated just recently (Thorner et al., 1982; Rivier et al., 1982), and of the inhibiting factor somatostatin (SRIF). Both peptides are modulated in turn by various biogenic amine systems within the hypothalamus as well as by other peptides (fig. I.1.).

There are no indications that the principles of the regulatory mechanisms during fetal life are in any way different from those found in later life. A schematic representation of the complex regulation of GH is given in fig. I.2..

GH can be detected in fetal plasma by 7 weeks of gestation. Its concentration rises from 65 $\pm$ 7 ng/ml at 10-19 weeks of gestation to a peak of 132 $\pm$ 22 ng/ml at 20-29 weeks, which is probably the result of a relatively autonomous secretion. Subsequently, these acromegalic values decline. This coincides with the development of the hypothalamo-pituitary system, when the regulatory mechanisms, both stimulatory and inhibitory, become operative, as well as with further maturation of the central nervous system (Gluckman et al., 1980). At birth, the GH regulatory mechanism is not fully developed yet, as is shown in the following observations (Goodyer, 1981):

- basal GH values are elevated and a diurnal rhythm fails to unfold till up to 3 months post-partum;
- hyperglycemia and L-Dopa or pyridoxin administration result in a paradoxical GH response during the first week of life.

I.4. The somatomedin concept

I.4.1. Sulphation factor - somatomedin - insulin-like growth factor

In vitro, serum from intact or GH-treated, hypophysectomized rats stimulates the incorporation of radioactive sulphate into cartilage of hypophysectomized rats. Neither GH itself nor serum from untreated, hypophysectomized rats with or without added GH has an appreciable effect on cartilage sulphation in vitro. This basic observation, made by Salmon and Daughaday, has led to the hypothesis that GH does not act directly on cartilage, but elsewhere in the body to stimulate the formation of a secondary hormonal mediator acting directly on cartilage (Salmon and Daughaday, 1957). This mediator was initially termed sulphation factor. As this factor seemed to mediate at least some of the anabolic effects of the somatotropic hormone (GH), it has been renamed somatomedin.

In the early sixties, it became evident that the insulin-like activity (ILA) of serum - defined as the ability of serum to stimulate metabolic activities in muscle and adipose tissue in vitro as crystalline insulin does - only partly consists of pancreatic insulin. The ILA of human serum stays present despite an excess of insulin antibodies. This nonneutralizable or nonsuppressible ILA (NSILA) constitutes the bulk ($\geq$ 90%) of ILA in fasting human serum (Jakob et al., 1968). Subsequently, it has been observed that partially purified SM exhibits insulin-like activity in various bioassays and that SM and ILA are co-purified in such a way that the two bioactivities maintain a fairly constant ratio. These and other observations point to a close similarity, if not identity of SM and NSILA in human serum. This identity has been validated by Rinderknecht and Humbel who discovered the aminoacid sequence of two peptides, for which they proposed the generic names insulin-like

growth factor (IGF) I and II. IGF-I and IGF-II have a 62% aminoacid sequence identity and are 38-48% homologous with the A and B domains of human pro-insulin (Rinderknecht and Humbel, 1976, 1978[a], 1978[b]). Several types of SM peptides have been isolated from human plasma, termed differently depending on the assay used [x1)], namely SM-A, SM-C, IGF-I, IGF-II and ILAS, all having a molecular weight of 7,000-9,500 and an isoelectric point range of 4.4-9.8 (Van den Brande and Hoogerbrugge, 1980; Guyda et al., 1981[b]) [x2)]. As more structural information is being acquired, several of the peptides may prove to be identical (Klapper et al., 1983) (table I.1.). A summary of in vitro and in vivo actions of SM is presented in tables I.2. and I.3. (after Phillips and Vasiloupoulou, 1980). SM levels in plasma are in accordance with the original definition of GH dependence, low in patients with GH deficiency and high in acromegalics.

x[1])
Bioassays refer to the biologic activity of a test sample in comparison with a "standard" preparation (hence the term somatomedin activity (SMA)). Various in vitro bioassays have been developed, based on the different tissue effects of SM (table I.2.). The porcine cartilage bioassay, by which the incorporation of radiolabeled sulphate into cartilage is measured, is most frequently employed (Van den Brande and Du Caju, 1974[a]).
In radioreceptorassays (RRA), competitive protein binding assays (CPBA) and radioimmunoassays (RIA), displacement of radiolabeled somatomedin is measured from cell membrane preparations, crude carrier protein fractions or antibodies respectively. The sensitivity and specificity of these tests vary considerably (for a review the reader is referred to Phillips and Vasiloupoulou, 1980, and Guyda et al., 1981[b]).

x[2])
With the exception of NSILP, a large molecular weight NSILA which cannot be converted into small fractions under acidic conditions and does not cross-react in a CPBA for NSILA (Poffenbarger et al., 1979; Zapf et al., 1978).

Exceptions to this rule have been observed. In addition, there are indications that the modulation of insulin secretion and the nutritional status play a major role in the regulation of the secretion of SM peptides (Phillips et al., 1976, 1979[a], 1979[b]; Price et al., 1979) (fig. I.2.). In 1978, Van Buul-Offers and Van den Brande showed that a somatomedin preparation, devoid of anabolic hormones, produces body growth in the congenitally GH-deficient Snell mouse to the same degree as growth hormone does (Van Buul-Offers et al., 1979). These findings, recently confirmed by Schoenle using pure IGF-I and IGF-II preparations (Schoenle et al., 1982[a,b]), strongly indicate the original hypothesis of Salmon and Daughaday to be correct.

I.4.2. Somatomedin binding proteins

There is ample evidence that SM circulates in plasma, mainly bound to macromolecular carrier or binding proteins (a.o. Zapf et al., 1975; Hintz and Liu, 1977; Furlanetto, 1980). When human plasma or serum is chromatographed on Sephadex at neutral pH, SM migrates as a large molecule; at acid pH, however, as a small molecule. At least two different binding proteins with high affinity and specificity for SM exist. The major one (150-200,000 MW) consists of two protein subunits, one of which is acid-stable and contains the structural determinants necessary for binding SM, while the other is acid-labile (Furlanetto, 1980). The acid-stable subunit appears to be under GH control (a.o. Zapf et al., 1980; Copeland et al., 1980; White et al., 1981; Schalch et al., 1982). Moreover, plasma contains an unsaturated, acid-stable form of an SM binding protein with a molecular weight of 35-40,000. Levels of this unsaturated SM binding protein are elevated in serum of patients with growth hormone deficiency and decline during GH treatment (Hintz et al., 1981; Borsi et al., 1982). Preliminary evidence has been presented, suggesting that

Table I.1.
Somatomedin peptides prepared from human plasma (after Guyda, 1981).

SM peptide	MW	p I	sulphation	growth effects	bioassay used for isolation
IGF-I	7649	8.2-8.5	+	+	^{14}C gluc. uptake into rat adipocytes
SM-C	7649	8.2-8.5	+	+	$^{35}SO_4$ and ^{3}H-thymidine uptake into rat cart.
IGF-II	7471	7.0-7.4	+	+	^{14}C gluc. uptake into rat adipocytes
SM-A	7000	7.1-7.5	+	+	$^{35}SO_4$ uptake into embr. chick cart.
ILAS	9400	6.4-6.7	+	+	^{14}C gluc. uptake into rat adipocytes
NSILP	88.000	5.9-6.5	-	+	^{14}C gluc. uptake into rat adipocytes

Table I.2.
Tissue effects of somatomedins (after Phillips et al., 1980)

Cartilage-stimulating activity
- amino-acid transport
- synthesis of RNA
- synthesis of DNA
- synthesis of protein
- formation of proteoglycan
- synthesis of collagen

Insulin-like activity

Muscle
- amino-acid transport
- sugar transport
- formation of glycogen
- synthesis of protein

Fat
- sugar transport
- glucose oxidation to carbon dioxide
- glucose incorporation into lipid
- inhibition of lipolysis

Mitogenic activity
- replication in cell cultures

Table I.3.
Demonstrated effects of SM in vivo (after Phillips et al., 1980)

Insulin-like activity

Muscle
- sugar transport
- glucose uptake into glycogen
- protein synthesis

Fat
- glucose incorporation into lipid
- decreased lipolysis

Whole animal
- increased glucose turnover
- hypoglycemia
- growth promoting activity

alkaline treatment of serum Cohn fraction IV results in a breakdown of the large molecular weight binding proteins into smaller complexes of approximately 40,000 and 30,000 MW (Morris and Schalch, 1982). The binding proteins are probably generated in the liver. Binoux recently provided evidence of the existence of two binding proteins in culture media of adult livers. Both have a molecular weight of approximately 40,000, but a different affinity for IGF-I and IGF-II (Binoux et al., 1982).

I.4.3. Somatomedin inhibitors

Under catabolic conditions, such as acute or chronic starvation and diabetic ketoacidosis, low values of SM are found in humans as well as in rats (Grant et al., 1973; Van den Brande and Du Caju, 1973; Phillips et al., 1976; Hintz et al., 1978; Clemmons et al., 1981[b]; Merimee et al., 1982[a], Isley et al., 1983). The relationship between SM and other hormones is unclear. Starvation results in elevated GH levels, an inability of administered GH to raise serum SM levels and a peripheral resistance to the action of GH (Phillips et al., 1976; Hintz et al., 1978). No significant

correlation has been found between SM-C and decreased insulin values during fasting and refeeding of adults (Isley et al., 1983). During starvation, glucose utilization by the periphery is reduced and fatty acids and aminoacids are primarily used for fuel expenditure rather than for de novo protein synthesis. Inhibition of peripheral glucose uptake is a typical property of GH. Low SM values, therefore, seem to permit the utilization of mobilized substrate for fuel homeostasis rather than for cell growth and proliferation. Thus, Merimee postulated that changes in insulin concentrations modulate the mobilization of substrate during fasting, whereas changes in GH and SM modulate the substrate pattern of usage (Merimee et al., 1982[a]). What causes the decrease of SM values during starvation is not exactly understood. Serum of starved, ketoacidotic patients displays inhibitory activity of SM action in various bioassays (Van den Brande et al., 1973; Salmon, 1975; Phillips et al., 1979[a], 1979[b]). This inhibitory activity is not due to glucagon, fatty acids or ketone bodies. Glucocorticoids are thought to exert no direct inhibitory activity, although recent evidence points out that corticosterone may inhibit the SO_4^- incorporation into hypophysectomized rat cartilage (Bomboy et al., 1983). The inhibitor, present in starved rat serum, is probably a nonpituitary-dependent, acid-stable, pH-dependent and heat-labile protein with a molecular weight of 27,000-40,000 (Salmon et al., 1983). This protein and the inhibitor, present in serum of diabetic rats, may be identical. The inhibition of SM activity cannot be explained on the basis of simple binding or inactivation of SM.

On the other hand, the presence of carrier proteins has been shown to alter the bioavailability of SM. The passage of the SM-carrier-protein complex through the walls of blood capillaries is restricted (Meuli et al., 1978). The metabolic activity of SM, when bound to binding proteins and meas-

ured by various bioassays, is reduced (Chochinov et al., 1977; Zapf et al., 1979; Drop et al., 1979; Knauer et al., 1980). Thusfar, the only documented function of SM carrier proteins is to increase the circulating half life of injected SM activity from a few minutes to several hours (Cohen and Nissley, 1976; Kaufmann et al., 1977). As outlined above, it is also possible that the SM binding proteins protect certain tissues from the unopposed biological activity of SM (Meuli et al., 1978; Zapf et al., 1979).

Knauer and Cunningham (1982) established a completely different function of SM carrier proteins: they reported on a carrier protein (CP) for epidermal growth factor (EGF). EGF is an SM-like peptide, originally extracted from male mouse submaxillary glands. CP possesses arginine endopeptidase activity and proteolytically processes pro-EGF (9,000 MW) to EGF (6,045 MW). In vivo and vitro, CP forms high molecular weight complexes with EGF (74,000 MW), composed of two EGF and two CP molecules. In addition, purified CP, free of contaminating EGF activity, has been shown to potentiate the proliferative response of human foreskin fibroblasts to EGF. Labeled CP binds to a single form of cell secreted protein, termed "carrier protein nexin" (CPN). This CP-CPN complex is bound to the cell surface via a receptor for the CPN portion of the complex and subsequently internalized.

In conclusion: SM binding proteins may be important determinants of biological effects of circulating SM.

I.5. Somatomedin and fetal growth

Various elements indicate that somatomedin is involved in the humoral regulation of fetal growth.

- First of all, there are the "experiments of nature". A decreased birth length is found in patients with Laron type dwarfism and in the African pygmy. Laron type dwarf-

ism is characterized by extremely short stature due to a defect in the GH receptors, which results in low serum SM values and high GH levels (a.o. Laron et al., 1971; Van den Brande et al., 1974). In the African pygmy, IGF-I deficiency has been demonstrated (Merimee et al., 1981). An increased birth length has been found in one infant with Beckwith-Wiedemann-Syndrome, with markedly elevated SM activity in cord blood (Ashton et al., 1978).

- The first stage in the action of peptide hormones, such as somatomedin, is their binding to specific receptor sites on the plasma membrane of their target cells. Specific receptors for radiolabeled SM have been detected in various human fetal tissues (Sara et al., 1983).
- SM concentrations are detectable in fetal serum. The pattern of serum SM-C concentrations in fetal sheep during gestation parallels the increase in fetal weight and crown-rump length (Handwerger, 1983).
- Levels of SM activity in cord blood correlate significantly with fetal and early postnatal growth (Brinsmead and Liggins, 1979[a]; Sara and Hall, 1980).
- Furthermore, fetal cells endogeneously produce somatomedins (D'Ercole et al., 1980[b]; Hill and Milner, 1981).
- Lastly, SM has a direct growth promoting effect on fetal cells in vitro (Sara et al., 1981; Hill et al., 1983; Kaplowitz et al., 1982[b]).

I.6. SM levels in fetal body fluids

Ethical standards obviously place restrictions on the investigation of fetal and perinatal endocrine physiology in humans. Measurements of SM activity, therefore, have been performed partly in amniotic fluid, but mostly in cord serum, in the expectation that they should reflect fetal levels rather than changes specific to parturition. Low SM bioactivity was found in amniotic fluid (Anderson et al., 1974;

Bala et al., 1976, 1978; Hill and Milner, 1981). It was higher at term than in early pregnancy. SM radioreceptorassay activity was high in preterm amniotic fluid and low in term samples (Moberg et al., 1976). However, both Chochinov and Drop, using a radioreceptorassay for SM as well, independently have established the presence of a binding protein for SM. No small molecular weight SM-like peptides have been demonstrated (Chochinov et al., 1977; Drop et al., 1979). The levels of this binding protein in amniotic fluid were highest at mid gestation; their subsequent decrease correlated with fetal renal maturation, suggesting a fetal origin of the binding protein (Chochinov et al., 1976).

It has been puzzling why SM activity in cord blood, whether measured by (postnatal) cartilage assay, radioreceptorassay or radioimmunoassay, is low in comparison with serum of adults, while simultaneously GH values are elevated and body growth and skeletal maturation rapidly increase during the last trimester (Sara et al., 1980; Goodyer et al., 1981; Hill and Milner, 1981). Hill and Milner (1981) investigated the following three explanations:

a. Hormonal influence other than that of SM on fetal cartilage

   Physiological or pharmacological doses of purified hormones, such as insulin, GH, PRL and MSH, did not promote growth of fetal human or rabbit cartilage. Fetal rat cartilage was stimulated to an equal extent by plasma from hypophysectomized adult rats as well as from intact rats. The stimulatory activity of the hypox-rat plasma was considerably reduced in cartilage obtained 4 days postnatally.

   In conclusion: fetal rat cartilage responded to a non-growth hormone dependent factor in hypox-rat plasma to which postnatal cartilage was refractory.

b. Detection of the predominant forms of fetal SM in assays for postnatal SM

SM activity was measured in fetal rabbit serum by rabbit cartilage bioassay with cartilage obtained from fetal and adult rabbits. In the postnatal cartilage assay, the activity was less than half of that found in the fetal rabbit cartilage assay. Fetal cartilage, therefore, recognized stimulatory factors in fetal plasma which were not detected in the postnatal cartilage. Several recent observations made in rats have suggested a possible role for IGF-II in fetal growth. IGF-II levels were 20-100fold higher in fetal rat serum than in maternal serum, declining within days after birth (Moses et al., 1980). IGF-I levels unfolded the reciprocal developmental pattern: low in the early neonate, rising to adult levels by approximately 4 weeks of age (Daughaday et al., 1982[b]). Fibroblast cultures from rats of 2 to 50 days of age mimicked the developmental switch from IGF-II to IGF-I production observed in serum (Adams et al., 1983).

Sara measured SM activity in human serum by radioreceptorassay, utilizing human fetal brain plasma membrane as matrix (Sara et al., 1981). Levels in fetal serum were found to be increased, not only compared with adult values, but also with SM values measured by radioimmunoassay. On the basis of this discrepancy between SM values measured in fetal serum by RRA and RIA, it was postulated that in the human fetus an embryonic form of SM exists. At the end of the first half of gestation, this embryonic SM was present in high concentrations. During the second half of gestation, the values gradually fell as adult forms of SM began being produced. In this study, however, native serum was tested and fetal serum was not subjected to another radioreceptorassay. Consequently, interference of binding proteins was not taken into account. D'Ercole was first to suggest the presence of

binding proteins in fetal serum (D'Ercole et al., 1980[d]). He reported on the elution profile of plasma of preterm newborns (of 27 weeks of gestation or less). The immunoreactive SM-C eluted predominantly at an apparent molecular weight of 40,000. Infants of 30 weeks of gestation or more showed an elution profile similar to that of postnatal sera (elution at a molecular weight of approximately 150,000). One anencephalic infant of 43 weeks of gestation exhibited only a 40,000 MW SM, suggesting GH or other pituitary hormone dependence (D'Ercole, 1980).

MSA (multiplication stimulating activity) is an insulin-like growth factor, isolated and purified from serum-free medium conditioned by a rat liver cell line. It is now considered to be the rat counterpart for IGF-II: finding high levels, Moses tested Sephadex-G75 chromatographed fetal rat serum in three different assays: radioimmunoassay, rat liver membrane radioreceptorassay and competitive binding protein assay (Moses et al., 1980).

In conclusion: In respect of a human form of embryonic SM, the data at present are inconclusive.

c. Cartilage sensitivity in the fetus

The sensitivity of rib cartilage in vitro to a fixed SM stimulus was found to be higher in late fetal life than postnatally (Hill and Milner, 1981). Human fetal circulating mononuclear cells had twice as many receptors for SM as adult cells (Rosenfeld et al., 1979).

In conclusion: A rapid fetal growth rate may partially be the result of an increased tissue receptivity to SM.

I.7. The origin of somatomedin in the fetus

SM in the fetus does not appear to be of maternal origin, since it does not cross the placenta (D'Ercole et al., 1980[a]) In adults, the liver has been implicated as the major site

of production. So have been kidney and muscle (McConaghey and Dehnel, 1972; Hall and Bozovic, 1969). Not only cultured human fibroblasts are recently shown to synthesize SM (Atkinson et al., 1980; Clemmons et al., 1981), but several fetal tissues release immunoreactive SM in culture as well (D'Ercole, 1980[b]). Therefore, D'Ercole proposed the hypothesis that the primary actions of somatomedin take place at its sites of origin (D'Ercole et al., 1980[b]).

I.8. The hormonal control of somatomedin in the fetus

At present, the SM regulatory system during fetal growth is largely unknown. Following decapitation or hypophysectomy in utero, body growth and skeletal maturation proceeded normally. In several animal studies, somatomedin activity proved not to be reduced despite the absence of immunoassayable GH (Hill and Milner, 1981; Brinsmead and Liggins, 1979; Daughaday et al., 1979). Factors other than GH have been investigated. Insulin administration in utero increased SM activity in the fetal circulation (Hill and Milner, 1981).

There is limited information about the influence of nutrition on SM in fetal animals. SM bioassay activity was decreased in serum of fetal guinea pigs with intrauterine growth retardation due to uterine artery ligation. However, the SM concentration measured by radioimmunoassay was higher than in serum of normal fetal guinea pigs (Lafeber, 1981). Recent data, acquired in young weaned rats, have suggested that caloric intake and especially protein content of the diet influence serum SM concentrations (Prewitt et al., 1982). Similar results were obtained in fasting and refeeding studies in human adults (Clemmons et al., 1981; Isley et al., 1983).

Underwood proposed the hypothesis that placental lactogen (PL) is the primary regulator of SM in the fetus (Underwood et al., 1979). Ovine placental lactogen was found

to be equipotent with ovine GH in raising SM levels in serum of hypophysectomized rats (Hurley et al., 1977). hPL, also known as human chorionic somatomammotropin (hCS), and hGH show an 85% homology in terms of aminoacid sequence (Bewley and Li, 1974). hCS is secreted by the syncytiotrophoblast into the maternal circulation, and only minute fractions end up in the fetal circulation (Baumann, 1979). Therefore, it seems unlikely that hCS regulates fetal SM

Table I.4.
Major functions of the human placenta (after Deal, 1981)

I. Transport (by ultrafiltration, gradient diffusion, carrier-mediated active transport, phagocytosis, pinocytosis)
- Substrates
  - sugars
  - amino acids
  - fatty acids
- Other nutrients
  - vitamins
  - trace metals
  - minerals
  - electrolytes
  - H_2O

II. Hormone synthesis
- Steroid hormones
  - progesterone
  - estrogens (E_1, E_2, E_3)
- Protein hormones
  - placental lactogen
  - chorionic gonadotropin
  - chorionic thyrotropin
  - chorionic follicle stimulating hormone
  - chorionic corticotropin
  - ACTH and related molecules (αMSH, βLPH, βendorphin)

III. Respiration
- O_2/CO_2 exchange

IV. "Barrier" to host rejection of fetus

secretion directly. Very recently, however, a direct in vitro stimulatory effect of PL on IGF-II synthesis in fetal fibroblasts has been demonstrated (Adams et al., 1983). In addition, hCS, as a GH-like hormone, may very well influence the maternal fuel metabolism, a major factor in fetal growth (Kaplan and Grumbach, 1979).

In this respect, the crucial role of the placenta should be mentioned. A summary of its multiple functions is given in table I.4. (adapted from Deal, 1981). Placental tissue is noted for its rich source of receptors for insulin and various SM peptides. Specific binding has been found of la-

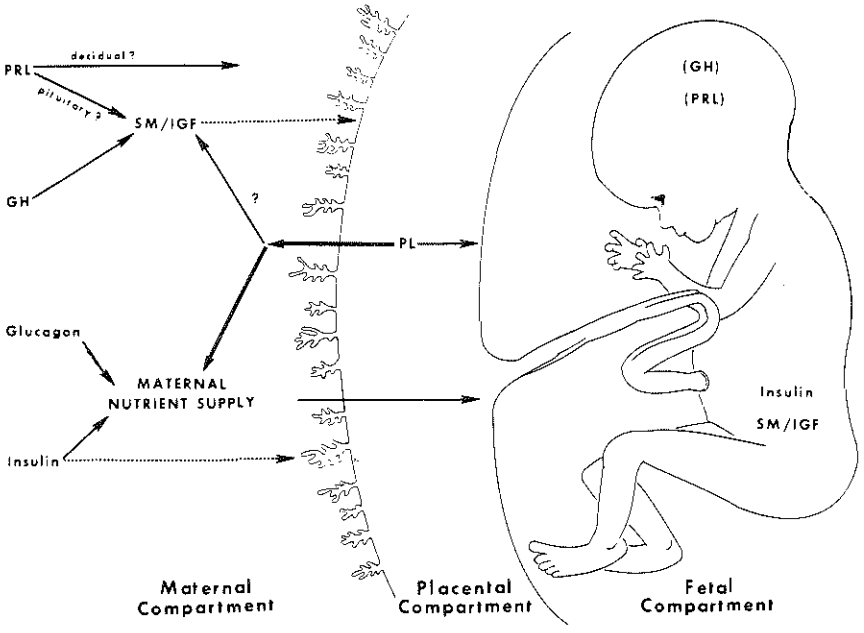

Fig. I.3.
Schematic diagram of hormonal determinants of fetal growth. Placental lactogen (PL) is a primary regulator of somatomedin in the fetus. In addition, growth hormone (GH), prolactin (PRL), the nutritional status and insulin almost certainly influence fetal somatomedin production (Underwood et al., 1979). (From: Deal, 1980. Thesis, McGill University, Montreal; reprinted with permission of the author.)

beled SM to cell membranes, prepared from cell cultures of human placentae obtained at early gestation and at term (Deal, 1982). These receptors are primarily localized on the microvilli of the syncytiotrophoblast and, therefore, would be responsive merely to maternal insulin (Nelson et al., 1978; Whitsett et al., 1979). Yet, in insulin-treated fetal rhesus monkeys, the placenta is shown to be increased in size despite normal maternal insulin levels (Susa et al., 1979).

In conclusion: Maternal and fetal SM may be potential regulators of placental metabolism.

I.9. Concluding remarks

The presence of growth promoting factors in the fetus has been recognized since the beginning of this century. Up to the present, embryonic extracts and fetal calf serum are being used to stimulate growth of cells in culture. The nature of the embryonic ingredients remains obscure (Sara et al., 1980). Understandably, somatomedin has been implicated as the actual fetal growth factor. As was pointed out, however, many questions in regard to its identity, origin, mode of action and regulation still go unresolved. A hypothetical and schematic representation of hormonal determinants of fetal growth is given in fig. I.3. (after Deal, 1981). Binding proteins may play a physiological key role in determining the bioavailability of SM acting primarily at its sites of origin (D'Ercole, 1980).

ACTA ENDOCRINOLOGICA 1979; 90: 505-518

CHAPTER II

PARTIAL PURIFICATION AND CHARACTERIZATION OF A BINDING PROTEIN FOR INSULIN-LIKE ACTIVITY (ILAs) IN HUMAN AMNIOTIC FLUID: A POSSIBLE INHIBITOR OF INSULIN-LIKE ACTIVITY

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II.1. Introduction

The most rapid growth in human cell number and cell size occurs during the intra-uterine and immediate post-natal periods of life (Cheek, 1968; Winick, 1971; Winick & Noble, 1965). The factors regulating intra-uterine growth are poorly understood. Growth hormone (GH) from the maternal or fetal pituitary may not be rate limiting (Blizzard & Alberts 1956; Chez et al., 1970; Jost, 1947; Laron & Pertzelan, 1969; Rimoin et al., 1966; Van Assche et al., 1969; Wells, 1947), although definitive human fetal studies of pituitary secretion of GH and of prolactin (PRL) in vivo have not been performed.

The importance of the effect of other factors, including insulin and somatomedin (SM) (Chochinov & Daughaday, 1976; Daughaday et al., 1972; Van Wyk et al., 1974; Van Wyk & Underwood, 1975), on somatic and skeletal growth in the fetus remains speculative. However, the significantly retarded birth length observed in infants with Laron dwarfism (Laron & Pertzelan, 1969; Laron et al., 1968) suggests that somatomedin may affect fetal growth. The hormones or factors

which promote synthesis and secretion of somatomedin in the fetus are unknown. Somatomedin bioactivity is low in amniotic fluid (Bala et al., 1978). Somatomedin bioactivity (Svan et al., 1977; Tato et al., 1975) and somatomedin reactivity, determined by radioreceptorassay (RRA) or radioimmunoassay (RIA), are also decreased in cord blood (Heinrich et al., 1978).

Chochinov (1977) has described a protein in midterm human amniotic fluid that reacted in the somatomedin-C RRA by binding labeled SM-C and that was capable of inhibiting the stimulation of thymidine uptake induced by human serum and by rat SM in human fibroblast cultures (Chochinov et al., 1977). The significance of this amniotic fluid binding protein in relation to fetal growth is uncertain.

In this report, we confirm and extend the observations by Chochinov et al. (1977). Our studies have been carried out with an insulin-like somatomedin peptide (ILAs) that we have partially purified in our own laboratory (Posner et al., 1977, 1978). Using a radioreceptorassay for this peptide, we have been able to characterize and partially purify a protein from human amniotic fluid (AFBP) that specifically binds ILAs and inhibits its bioactivity in vitro.

II.2. Materials and methods

II.2.1. Radioreceptorassay (RRA) for insulin (INS) and insulin-like activity (ILAs)

The RRA procedure was identical to the methods employed for INS and ILAs, as described previously (Posner et al., 1977, 1978). AFBP is reactive in the ILAs-RRA, but because of nonparallel displacement quantitative results (reported as ng equivalents of porcine insulin (1 ng equivalent equals 25 μU insulin)) should be taken as an approximation only.

In order to study the binding of AFBP to the placental receptor, the ILAs-RRA was modified as follows: in each assay tube, 0.1 ml of the placental membrane suspension and 0.1 ml of ILAs standard of AFBP were combined in a total volume of 0.5 ml of assay buffer (25 mM Tris, 10 mM $MgCl_2$, 0.1% BSA (Sigma), pH 7.4) and pre-incubated at $4^{\circ}C$ for 2 hours with continuous agitation. The incubation was stopped with the addition of 3 ml of ice-cold assay buffer. The tubes were then centrifuged at 750 x g for 30 minutes, the supernatant was decanted, and the membrane pellet was rinsed once with 3 ml of ice-cold buffer. The membrane pellet was then incubated with ^{125}I-ILAs (20,000 cpm) at $4^{\circ}C$ for 2 hours with continuous agitation, as in the usual assay conditions. Following the addition of 3 ml of ice-cold assay buffer, membrane bound radioactivity was separated by centrifugation and counted in a gamma spectrometer (Packard Model 5120, efficiency of 40%).

II.2.2. Purification of AFBP

II.2.2.a. Amniotic fluid (AF)

Amniotic fluid was collected per vaginam at the time of rupture of the membranes from normal pregnant women at term (AF_{term}). Early gestational AF ($AF_{preterm}$) was obtained for various diagnostic purposes by amniocentesis at a gestational age of 14-20 weeks. Any sample showing more than minimal blood contamination was discarded. The samples were pooled, frozen and stored at $-20^{\circ}C$ until processed. Upon thawing, the pooled fluid was cleared of particulate matter by filtration through cheese cloth, then through moarse filter paper and finally lyophilized and stored at $4^{\circ}C$ until used.

II.2.2.b. Acid-ethanol extraction

Lyophilized pooled AF powder was extracted with cold acid-ethanol (75% ethanol (v/v) - 0.19 M HCl, pre-cooled to 4°C). Lyophilized AF powder was homogenized in cold acid-ethanol (0.1 g/ml) in a Waring blender for 4 minutes and stirred at room temperature for 30 minutes. The suspension was then centrifuged at 30,000 x g for 30 minutes. The supernatant was dialyzed (Spectropor-3) against distilled H_2O for 48 hours and then against 0.01 M HCl for 24 hours. The dialysate was frozen and lyophilized.

II.2.2.c. Column chromatography

Gel filtration of lyophilized dialysate was performed on 5 x 100 cm columns of Sephadex G-150 (Pharmacia Fine Chemicals Inc., Uppsala, Sweden) at 4°C. The following buffer systems were used for purification:
0.01 M NH_4HCO_3, pH 7.4, and 0.01 M HCl, 0.1% bovine serum albumin (BSA) (Sigma), pH ~2. The elution volume of the amniotic fluid binding protein (AFBP) was determined by radioreceptorassay for ILAs (vide supra) on the eluted fractions. The void volume (V_o) and the total volume (V_t) of the column were determined with ^{125}I-labeled bovine thyroglobulin and ^{125}I respectively. Column fractions with the highest reactivity in the ILAs-RRA (Kd 0.4 to 0.5) were pooled and lyophilized.

II.2.2.d. Preparative isoelectric focusing

Preparative isoelectric focusing of AFBP, obtained following Sephadex chromatography, was carried out in a sucrose gradient (5-50%) stabilized column (LKB 440 ml) at 4°C. A

2% solution of Ampholine (pH 3-6) was employed. The anode solution consisted of 0.25 M H_2SO_4 in 80% (v/v) sucrose. The cathode solution was 0.25 M NaOH in water. After 72 hours, the column was emptied from below with continuous monitoring of absorbance at 280 nm. Eluate fractions were grouped and pooled according to pH and extensively dialyzed first against distilled water for 48 hours, then against 0.01 M HCl for 24 hours. Fractions with the highest specific activity (pH range 4.7 to 4.9) were lyophilized, redissolved in 0.01 M HCl and re-chromatographed on Sephadex G-150 in the same buffer (column size 2.2 x 40 cm).

II.2.2.e. Iodination

AFBP was iodinated by a modification of the chloramine-T method. Partially purified material (5-10 µg protein) was added to 0.5 M phosphate buffer, pH 7.4 (10 µl), 1.0 mCi Na-^{125}I (10-20 µl) and 40 µg of chloramine-T (25 µl). The reaction was terminated after 30-45 seconds with the addition of 250 µg of sodium metabisulphite (100 µl), followed by 100 µg of KI (100 µl). This mixture was chromatographed directly on a Sephadex G-150 column (2.2 x 40 cm) at 4°C, with 0.01 M HCl as eluting buffer. Only fractions eluting at a Kd of 0.4-0.5 were used for further studies.

II.2.3. Analytical procedures

II.2.3.1. Electrophoresis

Analytical polyacrylamide (disc-)gel electrophoresis (PAGE) was carried out with a 10% acrylamide separative gel, a 3% acrylamide concentrating gel and a continuous buffer system of Tris-glycine-HCl, pH 8.3. SDS-polyacryl-

amide disc-gel electrophoresis was performed as described by Laemli (1970).

II.2.3.2. Isoelectric focusing

Analytical isoelectric focusing was performed at 4°C in 7.5% polyacrylamide, 5% glycerol and 7 µM riboflavin-5'-phosphate containing 2% Amopholine (biolyte, Biorad, Cal.), pH range 3-6. The cathode solution consisted of 0.02 M $Ca(OH)_2$ and 0.04 M NaOH and the anode solution was 0.06 M H_2SO_4. Electrophoresis was carried out at 200 V for 18-22 hours. The gels were subsequently frozen on dry ice and sectioned into fractions with a Mickle gel slicer. Slices (3 mm) were incubated with shaking for 16 hours in 0.5 ml distilled water after which pH, ILAs-RRA reactivity or radioactivity of ^{125}I-labeled material was determined.

II.2.3.3. Molecular weight estimation

The molecular weight of AFBP was estimated by gel filtration on a Sephadex G-150 column (2.2 x 40 cm), calibrated in 0.1 M ammonium acetate, pH 7.4. As molecular weight standards the following proteins and peptides were chromatographed: aldolase 158,000; ovalbumin 45,000; chymotrypsinogen A 25,000; ribonucluolease (RNase) 13,700; parathyroid hormone 9,500; calcitonin 3,400.

II.2.3.4. Bioassays

Bioactivity of AFBP alone and AFBP in combination with ILAs was studied in chondrocyte cultures, prepared from rabbit growth plate and articular chondrocytes (Corvol et

al., 1978), as well as in the epididymal fat pad assay as described previously (Posner et al., 1978).

Protein concentration was determined by the method of Lowry et al. (1951), with bovine serum albumin (BSA) as the standard, or by optical density at 280 nm.

Statistical analysis was performed by the Student's t-test.

II.3. Results

II.3.1. Purification

Amniotic fluid binding protein (AFBP) was partially purified from batches of term and preterm AF by the following sequence of procedures: acid-ethanol extraction, gel filtration on Sephadex G-150 (repeated once), preparative isoelectric focusing and re-chromatography on Sephadex G-150. These procedures resulted in the isolation of samples of AFBP which had been purified from amniotic fluid about 825fold (table II.1.). Purification and recovery results should be taken as an approximation only, since the dilution curves in the ILAs-RRA of untreated amniotic fluid and AFBP at various stages of purity were not parallel to the ILAs standards. These relationships are more easily seen after logit-log transformation (fig. II.1.). Because of this, AFBP was assayed at the same protein concentration at each step of purification.

Preterm AF contained 7-10 times more ILAs-RRA displacing activity than term AF and was usually utilized as starting material. Lyophilized AF was extracted with acid-ethanol (0.1 g dry weight/ml), as described under Methods. This resulted in an approximately 7fold purification and removal of almost 90% of protein. Lyophilized dialysate

powder was dissolved in 0.01 M NH_4HCO_3 and then chromatographed on Sephadex G-150. The peak RRA activity eluted at a Kd value of 0.4-0.5, and peak reactive fractions were pooled and lyophilized. This step resulted in only slight purification with a low recovery. Subsequently, it was found that higher recovery could be obtained when AFBP was kept at acid pH. Therefore material obtained as above was re-chromatographed on Sephadex G-150 (column size 3.75 x 65 cm) in 0.01 M HCl, pH~2.

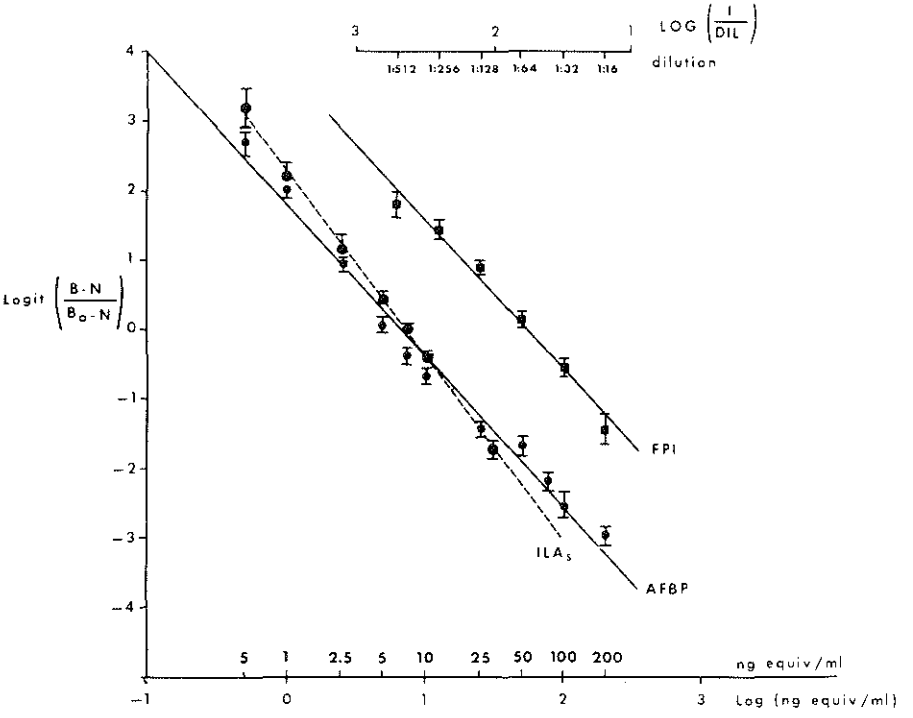


Fig. II.1.
Dilution curve of the ILAs standard, amniotic fluid (FPI) and partially purified AFBP in the ILAs-RRA. The data are shown after logit-log transformation. Unweighted linear regression analysis was performed upon logit-log transformed data.

Table II.1.
Purification schema

	Protein (Lowry) (mg)	RRA (ng equiv./ml)	Specific activity (ng equiv./mg)	Recovery (per cent)
1. AF 1250 ml	7875	1,025,000	130	100
2. Acid-ethanol extraction and dialysis	990	816,750	825	75
3. Sephadex G-150	230	263,120	1150	26
Repeated	73	232,960	3200	22
4. Isoelectric focusing	8.7	139,776	16,000	13
5. Sephadex G-150	0.78	83,865	107,000	6

Purification was now about 3fold, with a recovery of 89%, and RRA reactive fractions eluted at Kd 0.4-0.5, similar to the elution profile at neutral pH. Column fractions with highest activity from several chromatograms were pooled and lyophilized. In the next step, material obtained as above was subjected to preparative isoelectric focusing (see fig. II.2. and legend for details). This procedure resulted in a further 5fold purification and recovery was estimated at 59%. The fractions with the highest specific activity (SA) in the pH range of 4.7-4.9 were dialyzed, lyophilized and re-dissolved in 0.01 M HCl and chromatographed on Sephadex G-150 in the same buffer (column size 2.2 x 40 cm). A protein peak at the Kd value of albumin was clearly separated from RRA reactive material (data not shown). This procedure resulted in a further 7fold purification, with a recovery of 60%.

Table II.1. summarizes the results obtained throughout the entire sequence of procedures used for the isolation and partial purification of AFBP. The final recovery of RRA activity was estimated at about 6%. The apparent purification was 825fold. Analytical polyacrylamide disc-gel

electrophoresis in SDS of the final product showed one major protein band with an R_f of 0.44 (fig. II.3.).

II.3.2. Molecular weight

The molecular weight determination of AFBP was based on gel filtration on a standardized column of Sephadex G-150. In many successive runs of partially purified AFBP in neutral pH buffers, the Kd values were not significantly different and the combined Kd value was 0.435 $\pm$ 0.09 (mean $\pm$ SEM, n = 7). The range was from 0.403 to 0.472. This provides a molecular weight estimate of 34,500 daltons.

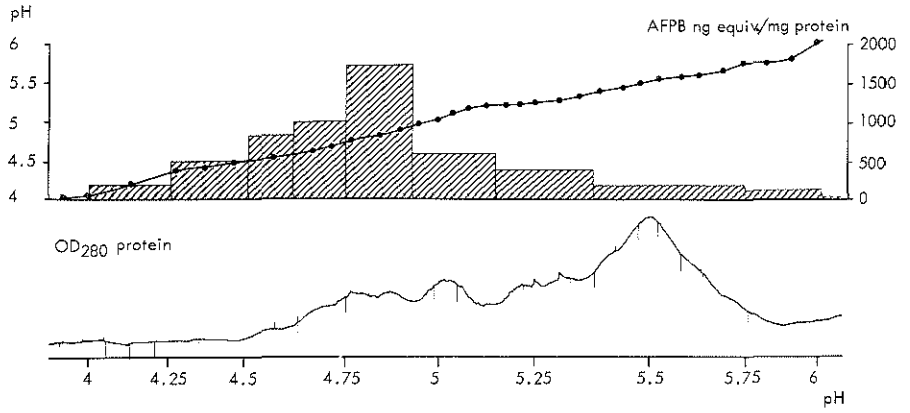


Fig. II.2.
Preparative isoelectric focusing. AFBP partially purified from term AF by acid-ethanol extraction and Sephadex G-150 chromatography (2X) with S A of 335 ng equiv./mg protein was dissolved in 18 ml H_2O (total amount of protein was 150 mg) and applied to a sucrose stabilized (5-50%) column (LKB 440 ml) at 4°C. Peak RRA activity in the eluted fractions measured after extensive dialysis was seen at pH 4.7-4.9. Bars represent S A in ng equiv./mg prot.
●——● pH gradient.
——— protein measured by OD at 280.

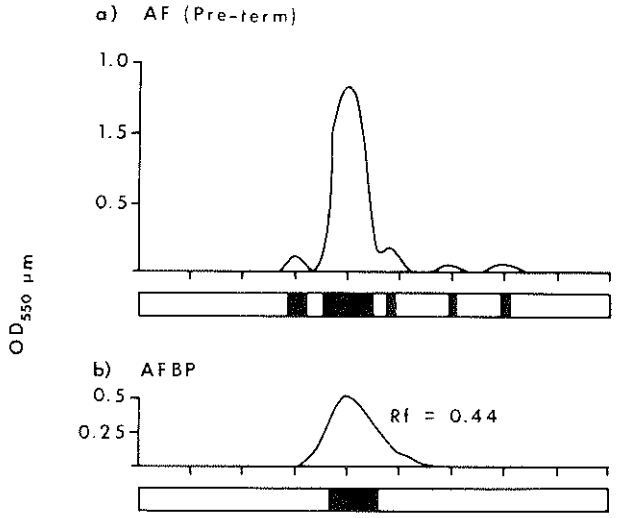


Fig. II.3.
Analytical gel electrophoresis in polyacrylamide gel (7.5%) with SDS.
a) The main protein band of pre-term amniotic fluid (albumin) had an R_f of 0.44.
b) Partially purified AFBP (100 ng equiv.) revealed only one protein band at R_f = 0.44.

II.3.3. Isoelectric point

As indicated in fig. II.2., during preparative isoelectric focusing the main RRA activity eluted at a pH of 4.7-4.9. When AFBP, labeled with ^{125}I as outlined under Methods, was subjected to isoelectric focusing in polyacrylamide gels, a major peak was seen at pH 4.6-4.9. A minor peak was found at 4.2-4.5, but it probably represents tailing of damaged tracer products as RRA activity was eluted only at pH 4.7-4.9 (not shown).

The nature of the interaction between AFBP and ILAs was defined by several different kinds of studies.

Linear Regressions		
H	Y-INT ± SEM	SLOPE ± SEM
INS	4 051 ± 0 124	−2 549 ± 0.070
ILA_s	3 682 ± 0.231	−2.286 ± 0.159
ILA_s + AFBP 25	5.038 ± 0.570	−2.432 ± 0.374
ILA_s + AFBP 75	6.506 ± 1.236	−2.282 ± 0.812

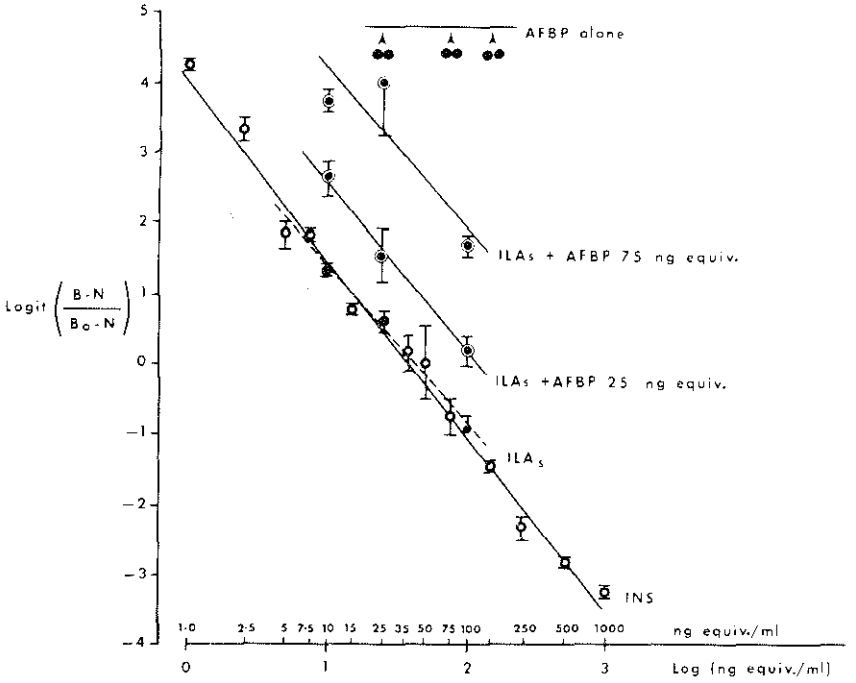


Fig. II.4.
Results of the assay of ILAs or of a mixture of ILAs and AFBP in the INS-RRA (after logit-log transformation). In the mixtures, concentrations of ILAs in the sample can be read off the x axis and concentrations of AFBP (in ng equiv./ml) are indicated along the corresponding regression line.

II.3.4. Interaction AFBP and ILAs

II.3.4.a. Radioreceptorassays

To exclude the possibility that the RRA reactivity of AFBP is based in part on direct binding of AFBP to the receptor, the ILAs-RRA was modified as outlined under Methods. Ninety ng equiv. AFBP (SA 1250 ng equiv./mg prot.), pre-incubated with placental membranes, did not inhibit ^{125}I-

Table II.2.
Binding of ^{125}I-ILAs to placental membranes after pre-incubation with ILAs or AFBP.

Final concentration in ng equiv. of porcine insulin (see Materials and Methods).

Peptide added	$\frac{B-N}{B_o-N}$
ILAs	
0.25	0.7621
1.0	0.7380
2.5	0.6294
4.0	0.5638
AFBP	
5	0.9900
15	0.9903
30	0.9952
90	1.050

ILAs binding to the receptor whereas 4 ng equiv. ILAs significantly decreased specific binding (table II.2.). In addition, ^{125}I-AFBP, incubated with receptor under usual assay conditions for 2, 4, 6 and 24 hours, did not reveal any specific binding. Finally, when a mixture of ILAs and AFBP was assayed in the INS-RRA, progressively higher concentrations of AFBP were observed to inhibit the activity of ILAs in the INS-RRA, although when assayed alone in the INS-RRA, AFBP was nonreactive (see fig. II.4. and legends for details).

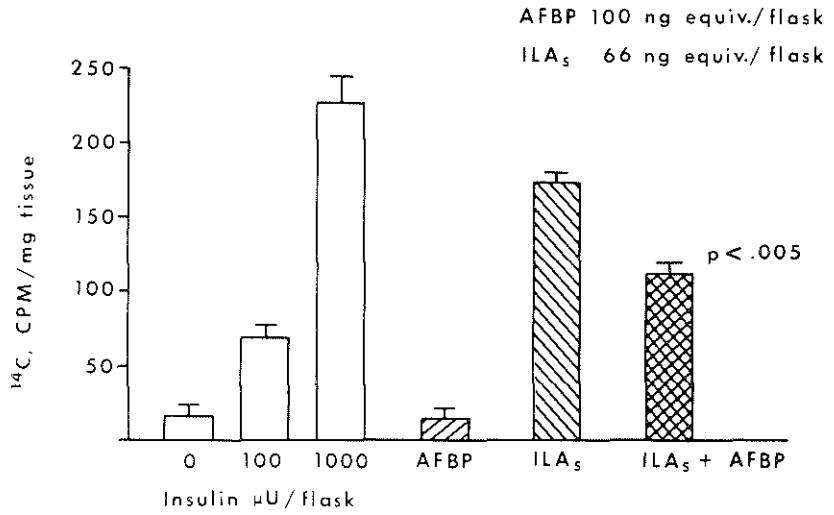


Fig. II.5.
Epididymal fat pad assay. The stimulatory effect of insulin on the incorporation of (^{14}C) glucose into fatty acids is expressed as ^{14}C CPM/mg tissue. Incubating AFBP (S A approximately 1000 ng equiv./mg prot.) at a concentration of 100 ng equiv./flask has no stimulatory effect. This amount added to ILAs (66 ng equiv./flask) decreased the stimulatory effect of ILAs alone significantly ($P < 0.005$).

II.3.4.b. Bioassays

AFBP significantly inhibited the stimulatory effect of ILAs on ^{14}C-glucose incorporation into fatty acids of rat adipose tissue (see fig. II.5. and legend). The increase of ^{35}S- sulphate incorporation into purified pro-

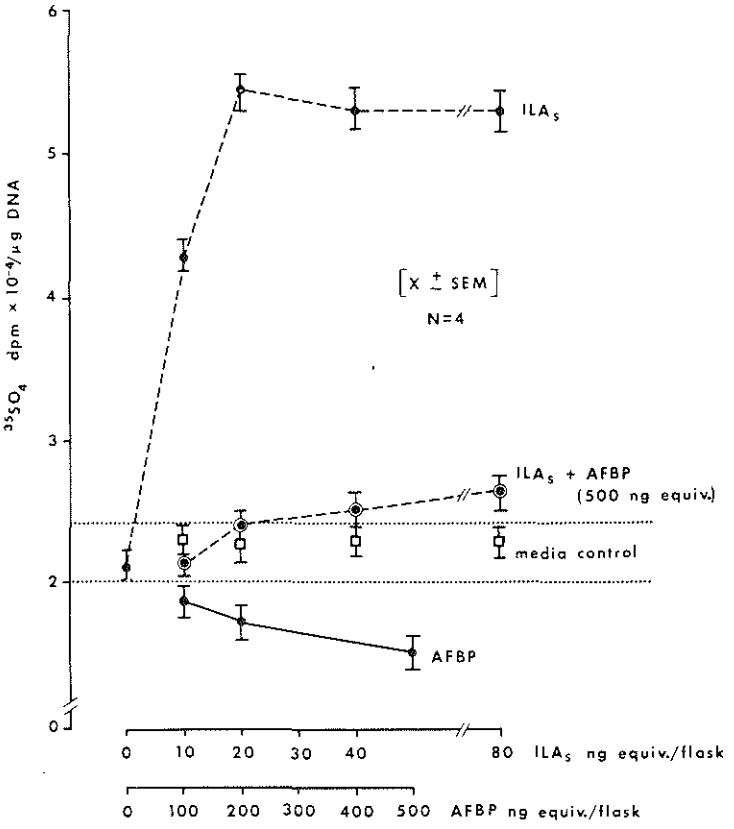


Fig. II.6.
Monolayer chondrocyte cell cultures prepared from rabbit growth plate and articulate chondrocytes were incubated for 20 h in Dulbecco's medium (Flobio, Courbevoie, France) without foetal calf serum. This medium was then replaced with sulphate-free Dulbecco's modified medium ($MgCl_2$ for $MgSO_4$) to which was added 1.5 µ Ci/ml $Na_2{}^{35}SO_4$ in the presence of different concentrations of ILAs, AFBP (S A 1250 ng equiv./mg prot.), or a mixture of both.

teoglycans of rabbit articular chondrocytes in monolayer culture produced by ILAs was completely blocked by AFBP (fig. II.6.).

II.3.4.c. Sephadex chromatography

Further evidence for the role of AFBP as a binding protein for ILAs is presented in fig. II.7.. AFBP was pre-incubated with ^{125}I-ILAs and chromatographed on Sephadex G-150 at neutral pH, resulting in a radioactive peak superimposable on the peak of AFBP as determined by ILAs-RRA (fig. II.7.a.). No binding of ILAs occurred at pH ~2 (fig. II.7.b.). When ^{125}I-ILAs and AFBP were pre-incubated in the presence of unlabeled ILAs, some radioactivity was now eluted at the Kd of ILAs (0.7). 10fold increasing the concentration of unlabeled ILAs resulted in the elution of most of the radioactivity at a Kd of 0.7. Unlabeled insulin (500 ng), added to the pre-incubation mixture, did not displace ^{125}I-ILAs from AFBP.

II.4. Discussion

Endogenous plasma ILAs is bound to large molecular weight proteins, and this binding is easily reversed by exposure to acid pH (Guyda et al., 1977). Gel filtration of amniotic fluid in 0.01 M HCl or in 1.0 M acetic acid - two procedures which have been uniformly effective in dissociating ILAs from binding proteins in plasma - has failed to demonstrate the presence of small molecular weight ILAs in human amniotic fluid. When lyophilized amniotic fluid powder was re-dissolved in 1/20th the original volume and chromatographed in 0.01 M HCl, thereby increasing the concentration 20fold, no ILAs-RRA reactivity was observed in fractions where ILAs

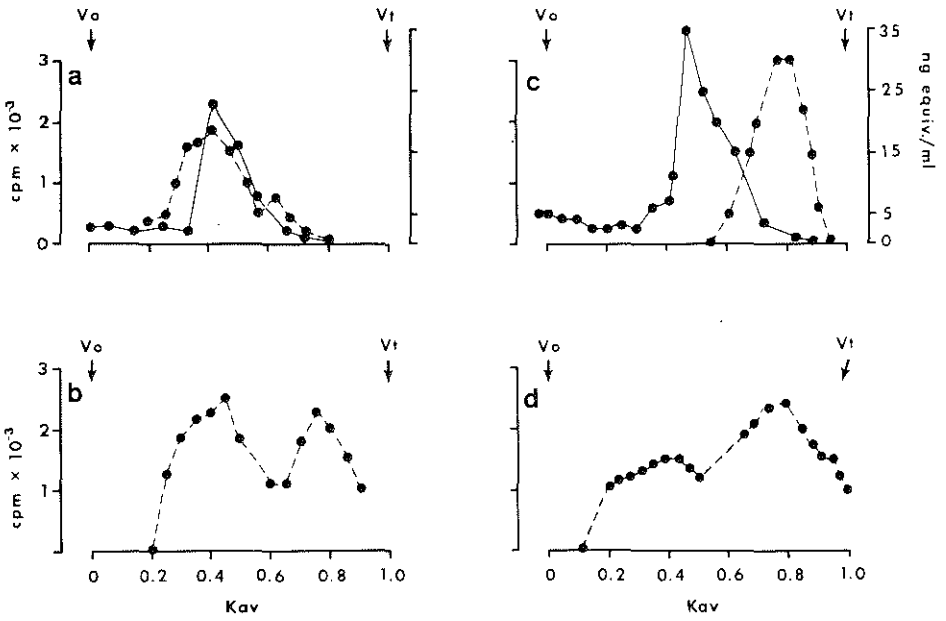


Fig. II.7.

Partially purified AFBP (S A 1250 ng equiv./mg prot.) was chromatographed on Sephadex G-150 (column dimensions 0.8 x 50 cm) at 4°C. 0.3 ml fractions were collected.

●——● RRA activity ●----● Radioactivity.

a. RRA activity was measured, after approximately 90 ng equiv. AFBP was dissolved and run in 25 mM Tris-HCl, 0.1% BSA buffer (pH 7.4). Pre-incubation with 150.000 CPM ^{125}I—ILAs at 4°C for 1 hour in the same buffer resulted in superimposable peaks.

b. Same quantities as in (a), but run in 0.01 M HCl (pH 2.2), show revers al of binding.

c. In addition to AFBP and ^{125}I-ILAs as above, 12 ng equiv. unlabelled ILAs was added to the pre-incubation mixture with buffer: 25 mM Tris-HCl, 0.1% BSA, pH 7.4.

d. Idem except 120 ng equiv. ILAs was added in the pre-incubation step.

would be expected. When incubated and chromatographed in 8 M urea, a procedure known to dissociate protein complexes, the ILAs-RRA reactivity, although diminished, was again recovered only in the large molecular weight region of the column. ILAs itself was stable in 8 M urea. It would, therefore, seem that the small molecular weight ILAs was absent from amniotic fluid and that all the ILAs-RRA activity could be accounted for by a moderately large (approximately 34 x 10^3 MW) acid protein (or proteins) we have termed AFBP. AFBP is heat-labile since more than 90% of its RRA reactivity was lost by heating to 100°C for 60 min.

The AFBP identified in amniotic fluid does not appear to be a protein-ILAs-complex, but it does display specific ILAs binding activity (fig. II.7.). Binding of ^{125}I-ILAs by AFBP was pH-dependent, dissociating at pH 2. Moreover, a dose-dependent inhibition of ^{125}I-ILAs binding was observed upon addition of various amounts of unlabeled ILAs. No inhibition of ^{125}I-ILAs binding by unlabeled insulin was observed under identical conditions, thus demonstrating the specificity of the AFBP-ILAs interaction.

To insure that AFBP did not inhibit binding of ^{125}I-ILAs to its placental receptor by competing for the binding sites, placental membrane binding of ^{125}I-ILAs was tested after prior exposure to AFBP. Binding of ^{125}I-ILAs was not inhibited by prior exposure of the membrane to AFBP. It would, therefore, appear that the ILAs-RRA activity of this protein was due to its ability to bind labeled ILAs, thus making it unavailable to the placental receptor.

Very little has been published on somatomedins or NSILAs in amniotic fluid. Bala et al. (1978) have reported that somatomedin, as measured by an in vitro hypophysectomized rat cartilage assay, was heterogeneous and consisted of at least three components. The major fraction, accounting for 86% of the total activity, had an apparent molecular

weight of more than 50,000 and did not dissociate into smaller molecules in 1% formic acid. The SM bioactivity was very low at midterm.

As our work was in progress, Chochinov et al. (1977) reported the results of their investigation of an amniotic fluid somatomedin binding protein using a SM-C-RRA. Their results closely parallel ours as to molecular weight, isoelectric point, nondissociation into smaller components and presence of the large molecular protein SM as the only detectable reactivity in the SM-C-RRA. Direct comparisons between our AFBP and Chochinov's SM-C-RRA reactive amniotic fluid protein (kindly provided by Chochinov) in our ILAs-RRA indicated similar behavior.

AFBP does not cross-react in the INS-RRA, even at a concentration of 500 ng equiv./ml, and one would predict that it would, therefore, be inactive in an insulin bioassay system. Such is indeed the case, since our material was not active in the epididymal fat pad assay. Furthermore, it inhibited ILAs activity in this bioassay system, presumably by binding ILAs. In addition, complete inhibition of sulphation activity by ILAs was observed in cultured rabbit chondrocytes. These data suggested that bound ILAs is not active with insulin tissue receptors, and they are in keeping with the observations that "bound insulin" or NSILAs is not physiologically active in vivo (Posner et al., 1968) or in vitro (Meuli et al., 1978).

Salmon (1972, 1975) has reported the presence of a somatomedin inhibitor in sera of hypophysectomized or starved rats and has postulated a physiological role for this inhibitor in limiting anabolic events, possibly to a greater extent than that resulting from the decrease of growth hormone release. Phillips (1979[a]) has also described a somatomedin inhibitor in diabetic rat sera that has a molecu-

lar weight between 10 and 60 x 10^3 daltons, estimated by Sephadex chromatography. These inhibitors could account for the dichotomy between growth hormone and somatomedin levels in human clinical conditions, such as the emotional deprivation syndrome or Kwashiorkor (Van den Brande & Du Caju, 1974; Van den Brande et al., 1975). The relationship between AFBP and these other SM inhibitors of plasma remains to be clarified. The development of a specific RIA for AFBP should facilitate its study.

CHAPTER III

FURTHER PURIFICATION OF AN AMNIOTIC FLUID BINDING PROTEIN

III.1. Introduction

From human plasma a number of peptides has been isolated, termed somatomedins, all having in common a GH dependency, a growth stimulating effect on cartilage and insulin-like activities (chapter I). These peptides, such as Insulin-like Growth Factor (IGF) I and II, Somatomedin-C, Somatomedin-A and Insulin-Like Activity (ILAs), have a molecular weight of 7,000-9,500 and circulate in serum, complexed to specific SM binding proteins (SMBP) with 150-200,000 MW (Zapf et al., 1975; Hintz and Liu, 1977; Furlanetto, 1980). In addition, plasma contains an acid-stable SM binding protein with ± 35,000 MW (Hintz et al., 1981; Borsi et al., 1982).

The precise function of these binding proteins is unclear. In addition to being carrier proteins, they may play an important role in the regulation of the biological effects of circulating SM (Meuli et al., 1978; Zapf et al., 1979).

From human preterm amniotic fluid, an acid-stable SM binding protein has been isolated (± 35,000 MW; pH 4.7), inhibiting SM-like activities in various bioassays (Chochinov et al., 1977; Drop et al., 1979).

The purification procedure, as outlined in our previous study (Drop et al., 1979: chapter II), proved to be cumbersome and time-consuming. The yield was insufficient in terms of recovery and purity to allow successful rabbit immunization and further characterization studies.

In this chapter, an improved procedure is described for the isolation of AFBP from preterm amniotic fluid, with high

speed gel filtration chromatography (HSGFC) and disc-gel electrophoresis as the final steps.

III.2. Materials

Human and bovine albumin was purchased from Sigma Chemical Col (St. Louis, Mo.). Sephadex and Sepharose came from Pharmacia Fine Chemicals (Uppsala, Sweden). 99.5% pure Tris was obtained from Koch Light Laboratories (Colnbrook, U.K.). Dextran-charcoal tablets were bought from Becton & Dickerson (Orangeburg, N.Y.).

Preterm amniotic fluid was obtained for diagnostic purposes by amniocentesis at a gestational age of 16-22 weeks (and kindly provided by prof. dr. M. Niermeijer, department of clinical genetics, Erasmus University, Rotterdam, and dr. J. Verjaal, department of genetics, University of Amsterdam). The samples were filtered (paper filter Mackeray-Nagel MN 640), pooled and kept frozen at -70°C until processed. Samples, macroscopically showing any blood contamination, were discarded.

III.3. Methods

III.3.1. Insulin-like activity

Insulin-like activity (ILAs) was purified as previously described (Posner et al., 1977). Recent studies with radioreceptor- and radioimmunoassays specific for IGF-I and IGF-II indicate that the neutral to slightly acid ILAs somatomedin peptide is similar to IGF-II, if not identical (Guyda et al., 1981).

III.3.2. Amniotic fluid binding protein charcoal binding assay (AFBP-CBA)

ILAs was iodinated by the chloramine-T method (Posner et al., 1977) (procedure kindly performed by W. Hackeng Ph.D., endocrine laboratory, Bergwegziekenhuis, Rotterdam).

AFBP concentrations were estimated by incubation of 100 µl of sample with 20,000 cpm ^{125}I-ILAs in a final volume of 500 µl with 25 mM Tris-HCl-1% BSA, pH 7.4. Incubation was performed for 2 hours at 4°C. Bound and free ^{125}I-ILAs were separated through addition of 500 µl of a 5.0% charcoal suspension in Tris-HCl-BSA buffer with 0.01% dextran. After 20 minutes, the charcoal was pelleted by centrifuga-

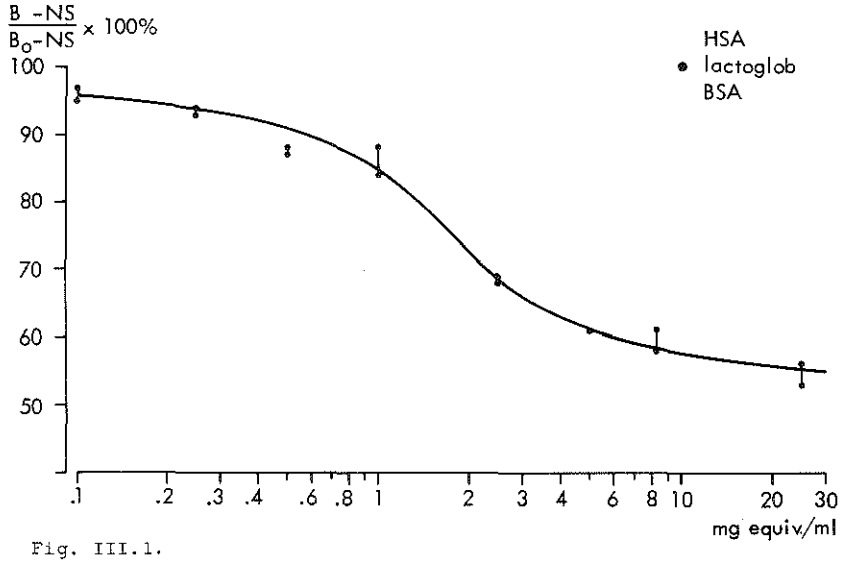


Fig. III.1.
Amniotic fluid binding protein charcoal binding assay (AFBP-CBA). Competitive-dose response curve for a crude AFBP preparation. In this figure, the AFBP content of acid-ethanol extract from preterm amniotic fluid is expressed in mg equiv. (protein)/ml and plotted against 100 x $\frac{B - NS}{B_o - NS}$, where B_o = ^{125}I-IGF-II, bound to dextran coated charcoal in the absence of AFBP, and B = ^{125}I-IGF-II, bound in the presence of AFBP. NS = non-specific binding.
Note: no displacement of HSA, BSA and lactoglobulin B.

tion at 3000 rpm at 4°C and the supernatant was discarded. ^{125}I-ILAs, adsorbed to the charcoal, was counted in a gamma-spectrometer (Searle, Chicago, Model 1195). Serial dilutions of an impure AFBP solution (acid-ethanol extract, see below) were chosen as the standard. The results were expressed in mg equiv. (protein)/ml. (fig. III.1.).

III.3.3. Protein determination

The protein concentration was determined by the Bio-rad protein assay (Bio-rad Laboratories, Cal.). The Bio-rad protein assay is a dye-binding assay based on the differential color change of a dye in response to various concentrations of protein. Bovine albumin was used as the standard.

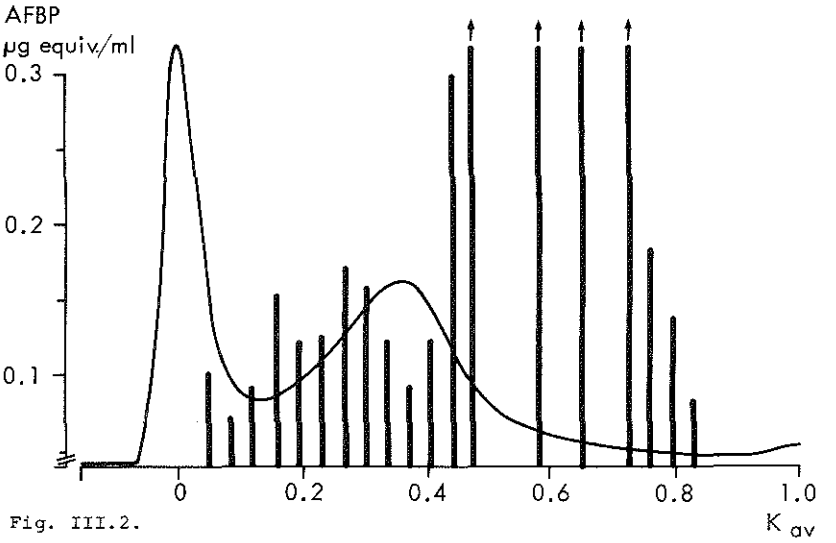


Fig. III.2.
Sephadex G-150 chromatography of concentrated acid-ethanol extract from preterm amniotic fluid. Buffer: 0.01 M HCl. Fractions were lyophilized and reconstituted into 25 mM Tris-HCl, 10 mM $MgCl_2$, pH 7.4, for AFBP-CBA determinations. For purification purposes the fractions between K_{av} 0.4-0.8 were pooled and lyophylized.
~ = OD_{280} ▮ = AFBP-CBA activity.

III.3.4. Acid-ethanol extraction and Sephadex G-150 column chromatography

Acid-ethanol extraction and Sephadex G-150 column chromatography were performed as previously described (Drop et al., 1979). Column fractions with highest activity in the AFBP-CBA were pooled and lyophilized (fig. III.2.).

III.3.5. High speed gel filtration chromatography (HSGFC)

Protein separation was achieved at room temperature on two protein analysis (30 cm x 7,8 mm ID) I-125 columns (Waters Associates, Milford, Mass.), connected in tandem with (0,009 in. ID) stainless steel tubing and zero dead volume fittings. The packing material in the columns consisted of 10 µ silica particles of 12.5 nm nominal size. A guard column (2 cm x 2 mm ID), containing packing material with 37-53 µ silica particles, was connected before the I-125 columns. The liquid chromatography assembly from Waters Associates (Milford, Mass.) consisted of one solvent delivery system (Model 6000 A) and a universal liquid chromatograph injector incorporated in the WISP 710 A. UV detection at 280 nm was performed with a model 440 absorbance monitor connected to a W-W recorder. Filtration of solvents was carried out with a pyrex filter holder and 0.5 µ filters from Millipore (Bedford, Mass.). Protein fractions were dissolved in 25 mM Tris-HCl, pH 7.0, and injected in 0.1-1 mg protein aliquots. The same buffer, at a flow rate of 0.5 ml/min., was used as mobile phase. The fractions containing AFBP were pooled and concentrated by centriflo membrane ultrafiltration (Amicon, Lexington, Ky.).

III.3.6. Disc-gel polyacrylamide gel electrophoresis

Disc-gel polyacrylamide gel electrophoresis was carried out at 4°C, with a 20% acrylamide separative gel, a 2.5% acrylamide concentrating gel and a continuous buffer system of Tris-glycine-HCl, pH 8.3. The proteins were eluted by a modification of the method described by Mendel-Hartvig (1982): following electrophoresis the gels (0.5 x 10 cm) were sliced into 0.5 cm fractions. These were reapplied on a separative gel. A stacking gel was formed on top of the sample gel. The tubes were sealed with dialysis membrane (MW cutoff at 10,000). When the poles were reversed, the protein was electrophoresed through the stacking gel in an overlay of Tris-glycine buffer. The overlay was removed with a syringe, pooled, dialysed and concentrated in a Micro-Prodicon negative pressure micro protein dialysis concentrator (Bio-Molecular Dynamics, Beaverton, Ore., USA).

III.3.7. Polyacrylamide slab-gel electrophoresis
III.3.8. Analytical polyacrylamide ampholine isoelectric focusing

Both polyacrylamide slab-gel electrophoresis, with and without sodium dodecyl sulphate (SDS), at pH 7.0, with a 12% separative gel and a 4% concentrating gel, and analytical polyacrylamide ampholine isoelectric focusing, with ampholine PAG plates (pH 4-6.5), were carried out on an LKB 2117 multiphor system according to the manufacturer's directions (LKB, Stockholm, Sweden).

III.3.9. Affinity chromatography

Affinity chromatography was performed on a Concanavelin A Sepharose column (2.7 x 11 cm) in 20 mM Tris-HCl, 0.5 mM

NaCl, pH 7.4 buffer. The proteins were eluted with methyl-alpha-D-mannoside (0.2 M) in the starting buffer.

III.3.10. Double immunodiffusion

Double immunodiffusion in gel was carried out by the method of Ouchterlony (1962), with gels prepared from 1.5% agar in 0.4 M Tris-barbital buffer, pH 8.8.

III.3.11. Staining procedures

III.3.11.a. Coomassie brilliant blue stain

Gels were soaked for 3 hours in 0.25% (W/V) Coomassie blue R-250, 45% (V/V) methanol, 10% acetic acid, then de-stained in 10% methanol, 7% acetic acid.

III.3.11.b. Silver stain

Gels were soaked for 1 hour in 30% methanol, 10% TCA and 3.5% sulphosalicilic acid, washed thoroughly in 15% methanol, then soaked in 5% glutardealdehyde and washed in 15% methanol and distilled water. The gels were stained with a solution of 0.7% $AgNO_3$ in 0.08% NaOH - 1.4% NH_4OH for 30-40 minutes. After washing in distilled water, the staining was terminated by incubation of the gels in 0.001% citric acid - 0.007% formaldehyde for 10 minutes.

III.3.12. Binding studies

Partially purified AFBP (Sephadex G-150 purified material, step 3, table III.1.), 100 µg equiv. prot./tube, was

pre-incubated for 2 hours at 4°C in 200 µl 10 mM Tris-HCl-0.5% BSA buffer (pH 7.4) with 100 µl ILAs (IGF-II) at various dilutions (0, 25, 50, 75, 100, 500, 750 ng equiv./ml). 100 µl of labeled SM-C/IGF-I or IGF-II respectively (+ 25,000 cpm) then was added. Following a further 2-hour incubation, 500 µl charcoal solution (1% norit A + 0.1% dextran T 250 in 10 mM Tris-HCl, pH 7.4) was added. After 20 minutes, the charcoal was pelleted by centrifugation at 3000 cpm at 4°C for 30 minutes and the supernatant was discarded. The pellet was counted in a gamma-spectrometer (Packard, Model 5120).

III.4. Results

III.4.1. AFBP charcoal binding assay (AFBP-CBA)

As the SM binding proteins present in preterm amniotic fluid are predominantly, but not exclusively small molecular weight proteins (30-50,000 MW range) (see fig. III.2.), the AFBP charcoal binding assay could be used as a simple and effective method to detect and quantitate a protein with an apparent molecular weight of 35-40,000 and SM (ILAs or IGF-II) binding activity. An impure AFBP solution (acid-ethanol extract, step 2, table III.1.) was designated as the standard. As shown in fig. III.1., no cross-reactivity was observed with either human or bovine serum albumin and lactoglobulin at a concentration of 1-10 mg/ml.

III.4.2. Purification

The purification steps are given in table III.1.. During the first step, acid-ethanol insoluble proteins, the large molecular weight SM binding proteins included, were pre-

Table III.1.
Purification schema

	mean specific activity (CBA) µg equiv./mg prot.	estimation of protein recovery %
1. AF pools (n = 5)	1,441	100
2. Ethanol-extract pools (n = 11)	1,064	52
3. Sephadex G-150 pooled fractions (n = 7)	15,741	28
4. HSGFC I + II (n = 3)	57,800	3.5
HSGFC III (n = 1)	202,300	1.5
5. Disc-gel electrophoresis	?	?

cipitated. The specific activity (SA) did not improve during this initial step, probably due to the fact that all SM binding proteins react in the charcoal binding assay causing spuriously high values in unpurified amniotic fluid. However, Sephadex G-150 column chromatography (fig. III.2.) resulted in a slight increase of SA. The effect of repeated HSGFC on the purification is illustrated in fig. III.3.a., b., c., d..

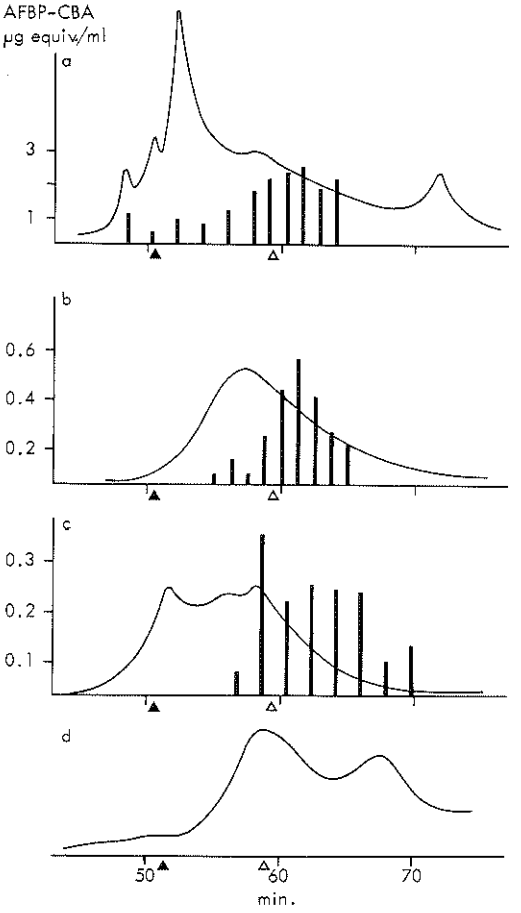


Fig. III.3.a.
Sephadex G-150 chromatographed material (see fig. III.2.) was subjected to high speed gel filtration chromatography (HSGFC I).
—— = OD_{280}, ▮ = AFBP-CBA activity, ▲ = HSA, Δ = lactoglobulin B.

Fig. III.3.b.3.c.
Fractions with highest AFBP-CBA activity were reapplied twice (HSGFC II, III).

Fig. III.3.d.
Note: following HSGFC III, no protein extinction (OD_{280}) at the elution volume of HSA with UV detection at highest sensitivity.

This procedure resulted in a further 30fold purification. As albumin, through the Ouchterlony immunodiffusion technique, was found to be still present as a contaminant (data not shown), fractions with highest activity in the AFBP-CBA were pooled and the freeze-dried material was subjected to disc-gel polyacrylamide gel electrophoresis. Fractions showing AFBP-CBA activity, but devoid of human albumin as determined by the Ouchterlony immunodiffusion technique, were pooled (fig. III.4.). AFBP was recovered by electrophoretic concentration according to a procedure described by

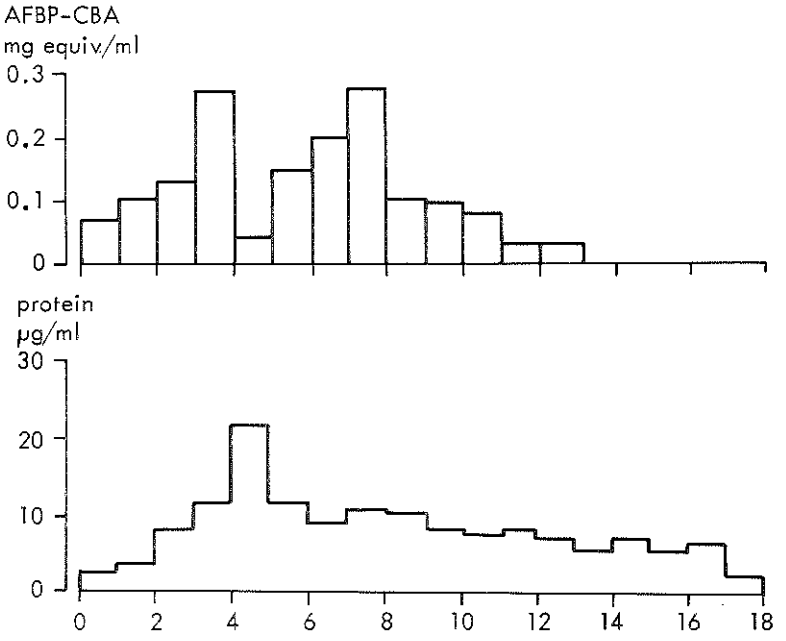


Fig. III.4.
Disc-gel polyacrylamide gelelectrophoresis. Protein fractions were recovered by a modification of the method described by Mendel-Hartvig (see materials). In this figure, the gel electrophoresis of Sephadex G-150 purified AFBP is shown because of limited supply of HSGFC purified material. At a gel length of ± 4 cm, highest protein concentrations are due to HSA, whereas highest AFBP-CBA activity is recovered at a gel length of ± 8 cm.

Mendel-Hartvig (1982), with a modification as outlined in Materials and Methods. An estimation of protein recovery is presented in table III.1.. It should be noted, however, that the capacity of the last two purification steps was very limited (see also appendix 1, chapter III). Therefore, an insufficient amount of purified AFBP was available for measuring charcoal binding activity and calculating specific activity. Priority was given to characterization studies (see below).

III.4.3. Binding studies

To determine the binding affinity of AFBP to IGF-I and IGF-II, a competitive binding assay was performed. Very poor displacement of labeled IGF-I and IGF-II by ILAs of up to 500 ng equiv./ml was found (studies kindly performed by H.J. Guyda, McGill University, Montreal, Canada). Partially purified AFBP (± 250 µg protein; step 3, table III.1.) was cross-linked with ^{125}I-SM-C in the presence of disuccinimidylsuberate (DSS) and run on polyacrylamide gels in the presence of unlabeled SM-C (100 and 800 ng respectively). Predominant binding took place at a Kf corresponding to an apparent molecular weight of 39,000. No displacement occurred in the presence of 800 ng SM-C (studies kindly performed by J. D'Ercole, University of North-Carolina, Chapel Hill, USA). Due to this lack of displacement, it was not possible to calculate an affinity constant by Scatchard analysis.

III.4.4. Bioassays

Inhibition of IGF-I/SM-C activity by partially purified AFBP (step 3, table III.1.) was demonstrated by Z. Laron

(Beilinson University, Tel Aviv, Israel) using a chick embryo cartilage bioassay (Silbergeld et al., 1981) (data not shown). Van Buul and Van den Brande (State University of Utrecht, the Netherlands) registered inhibition of SM (partially purified SM and plasma SM activity) in their porcine costal cartilage bioassay (fig. III.5.).

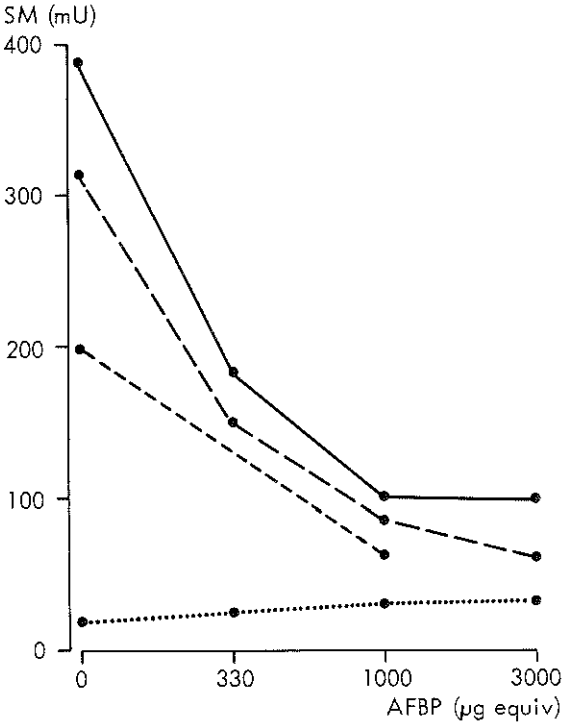


Fig. III.5.
Partially purified AFBP (step 3, table III.1.), at various concentrations (abscissa), was added to medium (·····), to 0.4 U plasma (———), 0.2 U plasma (— — —) and to 0.2 U plasma + 0.15 U of a somatomedin preparation (-----). AFBP inhibits SM activity (ordinate) measured in the porcine costal cartilage SM bioassay (Van den Brande and du Caju, 1974) (kindly performed by S. van Buul-Offers, Utrecht).

III.4.5. Characterization

Disc-gel purified AFBP was subjected to analytical slab-gel (SDS) polyacrylamide gel electrophoresis. Following a silver staining procedure of the gel, only one band was identified (fig. III.6.).

A previously determined isoelectric point of 4.7 (Drop et al., 1979) was confirmed by analytical isoelectric focusing. Disc-gel purified AFBP was run on an LKB Ampholine PAG plate (pH range of 4-6.5). Following silver staining of the gel, 3 bands in the narrow pH range of 4.7-4.9 were identified (fig. III.7.).

Disc-gel purified AFBP was subjected to analytical ultracentrifugation (kindly performed by W. van Noort and

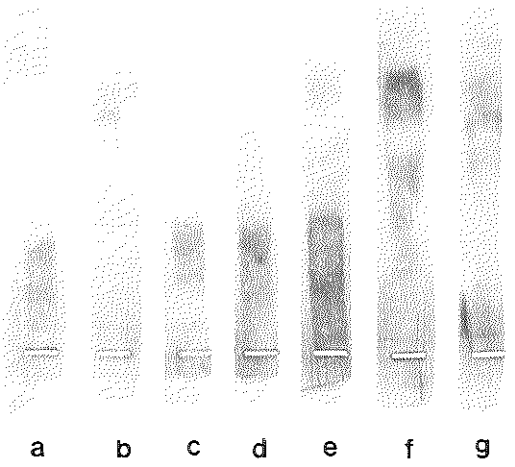


Fig. III.6.
Analytical slabgel SDS polyacrylamide gelelectrophoresis of AFBP containing fractions at various stages of purification. Applied amount of protein 1-10 µg per slot.
a = HSA; b = lactoglobulin B; c = amniotic fluid; d-g = purification step 2-5 (table III.1.).
AFBP is visible following step 3 (gel e). After disc-gelelectrophoresis one band is shown (gel g). The gels were silver stained.

prof. dr. H.G. van Eijk, department of chemical pathology, Erasmus University, Rotterdam). The initial studies were inconclusive, due to low protein concentration of the sample. Partially purified AFBP (step 3, table III.1.) was applied on a Concanavalin A Sepharose column. Following elution with methyl-alpha-D-mannoside, AFBP activity was recovered, which suggested AFBP to be a glycosylated protein.

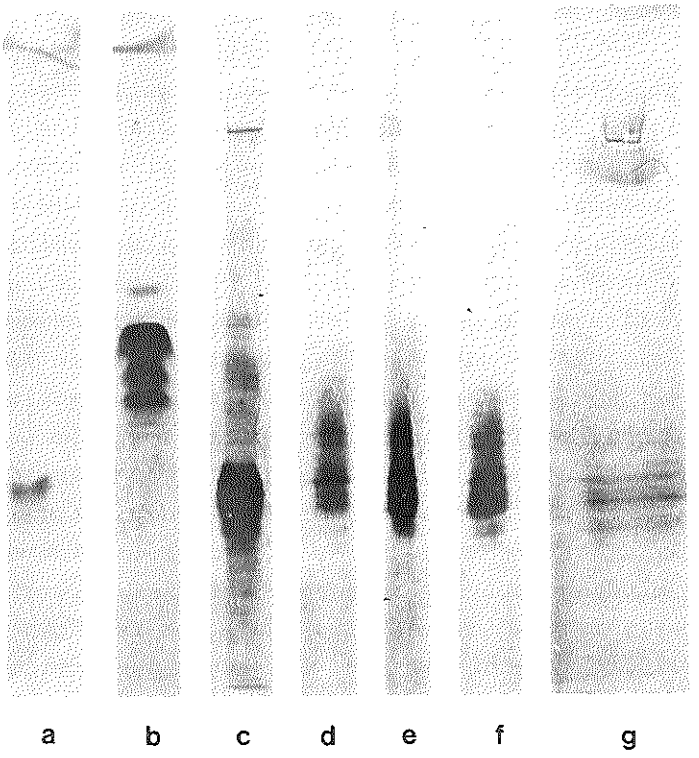


Fig. III.7.
Analytical iso-electric focusing (LKB Ampholine PAG plates, pH 4-6.5).
a = HSA; b = lactoglobulin B; c = amniotic fluid= d-g = purification step 2-5 (table III.1.).
Disc-gel purified AFBP is visible as multiple bands within a pH range of 4.7-4.9, gel g. The gels were silver stained.

The stability of AFBP was tested by subjection of partially purified AFBP (step 2, table III.1.) at various dilutions to a temperature of 20°C, 60°C and 100°C and to a pH range of 1.0-11. Details of this study are given in appendix 2, chapter III. The results indicate that the SM binding activity is maintained regardless of temperature and pH.

III.5. Discussion

Amniotic fluid binding protein was detected when in preterm amniotic fluid in an SM placenta membrane radioreceptorassay activity was found which could not be attributed to small molecular weight SM (Chochinov et al., 1976; Drop et al., 1979). At first, AFBP activity was determined in this RRA, but because of nonparallel displacement, quantitative results could be taken as an approximation only (Drop et al., 1979; chapter II). Preterm amniotic fluid mainly (but not exclusively) contains small molecular weight SM binding protein(s). Therefore, a simple and direct, albeit crude way of quantitation of an SM binding protein was found in the charcoal binding assay, similar to previously described methods for the determination of SM-C/IGF binding activity in serum (Moses et al., 1979; Zapf et al., 1980; Hintz et al., 1981). As is shown in fig. III.1., the sensitivity of the assay was limited. No further specific binding occurred with standard solutions of crude AFBP containing more than 10 mg protein/ml, possibly due to the high protein content and decrease of accessible binding sites (Daughaday et al., 1982[a]).

The purification of AFBP from preterm amniotic fluid was hampered by the following circumstances:

1) The AFBP content in preterm AF in terms of microgram protein is very low as, during disc- or slab-gel poly-

acrylamide gel electrophoresis of ethanol extract and Sephadex G-150 purified AFBP (step 2 and 3, table III.1.), no stainable protein bands were visible even with a very sensitive silver staining procedure, whereas SM binding activity could be demonstrated.

2) As far as the last purification step, albumin remained present and substantial losses could be accounted for by shoulder fractions and trailing (fig. III.2., III.3.).

   As both AFBP and albumin have an isoelectric point of 4.7-4.8, separation had to be based upon differences in chromatographic behavior due to molecular size. Therefore, high speed gel filtration chromatography proved to be a valuable tool in the purification (see also appendix 1, chapter III).

3) It is likely that low AFBP recovery during the final purification steps was also caused by nonspecific attachment of AFBP to tubing, dialysis membrane, glassware etc. On the other hand, one could speculate about an interaction between HSA and AFBP maintaining the stability of AFBP, as has been demonstrated for pre-albumin and retinol binding protein (RBP) (Kanai et al., 1968). Both binding proteins retained their binding potential since the pre-albumin-thyroxin binding as well as the RBP-Vit A binding were found to be independent of this protein-protein interaction (Raz & DeWitt Goodman, 1969).

Purified AFBP migrated as a single band in analytical polyacrylamide gel electrophoresis systems with an apparent molecular weight of 35-40,000.

AFBP probably is a glycoprotein as it is recovered from a Concanavelin A column. Therefore, the molecular weight determination may be subject to error. The multiple bands, found during analytical polyacrylamide ampholine isoelectric focusing at a pH range of 4.7-4.9, may repre-

sent heterogeneity in the degree of glycosylation of the AFBP molecule.

As discussed in chapter II, AFBP is acid-stable. In appendix 2, chapter III, it is outlined that the SM binding activity is heat-stable, irrespective of the pH.

In a competitive binding assay in which labeled IGF-I and IGF-II were tested, AFBP appeared to be an SM binding protein with low affinity and such high capacity that formal Scatchard analysis proved unsuccessful.

Thusfar, the binding proteins for peptides, which have chemically been fully characterized, are the MSA carrier protein, purified from a rat liver cell line (Knauer et al. 1981), and the EGF binding protein, purified from homogenates of submaxillary glands of male albino mice (Taylor et al., 1974). The MSA carrier protein consists of two different, non-covalently linked protein chains with an apparent molecular weight of 30,000 and 31,500. The EGF carrier protein has a molecular weight of 30,000, an isoelectric point of 5.6 and possesses arginine-endopeptidase activity.

Recently, two SM binding proteins with an apparent molecular weight of approximately 46,000 and 30,000 were demonstrated in human serum (Morris and Schalch, 1982). These proteins appeared to be breakdown products of an alkaline-treated, 60,000 MW SM binding protein complex. Hintz described an unsaturated form of an SM binding protein with a molecular weight of 35-40,000 (Hintz et al., 1981). Binoux suggested the existence of two binding proteins in culture media of rat and human liver cells, each with a molecular weight of approximately 40,000, but with different affinity for IGF-I and IGF-II (Binoux et al., 1982). As these serum binding proteins share several biological characteristics with AFBP, it would be of great importance to prove or disprove their homology.

The purification of AFBP permitted the immunization of

rabbits and the raising of specific antibodies against AFBP. The development of a radioimmunoassay for AFBP is presented in chapter IV.

APPENDIX 1,CHAPTER III

HIGH SPEED GEL FILTRATION CHROMATOGRAPHY

App. 1, III.1. Introduction

Gel filtration chromatography (GFC) has been widely used to separate proteins of varying molecular size. In the methods used to obtain these separations, cross-linked dextrans, polyacrylamide and agarose gels have been applied. With the development of rigid microparticulate packings, such as diol-modified silica gels (fig. app. 1,III.1.), with both low adsorptivity and low hydrophobicity, rapid separation, largely based on the effective size and shape of the protein, has become feasible (Majors, 1980). Hence,

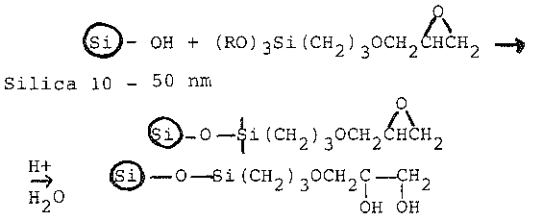


Fig. app. 1, III.1.
Reaction equation used in the preparation of the diol-modified silica gel (Majors, 1980).

this method has become known as high performance (speed)-size exclusion (molecular partition)-gel filtration.

As the parameters determining the extent and effectiveness of the separation may differ from one protein to the other (see table app. 1,III.1.), we performed a limited number of experiments in order to determine the most effective setup for the further purification of partially purified AFBP.

App. 1, III.2. Materials and methods

Human, bovine and ovine albumin and lactoglobulin B were purchased from Sigma Chemical Co. (St. Louis, Mo.). I-125 came from Radiochemical Centre (Amersham, U.K.). Filtration of solvents was carried out with a pyrex filter holder and 0.5 μ filters from Millipore (Bedford, Mass.). Protein fractions were dissolved in 25 mM Tris-HCl, 10 mM $MgCl_2$, pH 7.4, unless stated otherwise. Gel filtration was performed at room temperature on one and, later, on two I-125 protein columns (30 cm x 7.8 mm ID) (Waters Ass., Milford, Mass.). The packing material in the columns consisted of 10 μ silica particles of 12.5 nm nominal size, giving the column exclusion limits of 2,000 to 80,000 MW. A guard column (2 cm x 2 mm ID) contained packing material with 37-53 μ silica particles. The liquid chromatography assembly from Waters consisted of one solvent delivery system (Model 6000 A) and a universal liquid chromatograph injector incorporated in the WISP 710 A. Unless stated differently, UV detection at 280 nm was done with a Model 440 absorbance monitor, connected to a W/W recorder (Scientific Instruments, Basel, Switzerland).

App. 1, III.3. Results

The influence of various parameters on the resolving capacity of high speed gel filtration chromatography of human serum albumin (67,000 MW), ovalbumin (43,000 MW) and lactoglobulin B (35,000 MW) was investigated, before amniotic fluid binding protein (AFBP) fractions were tested.

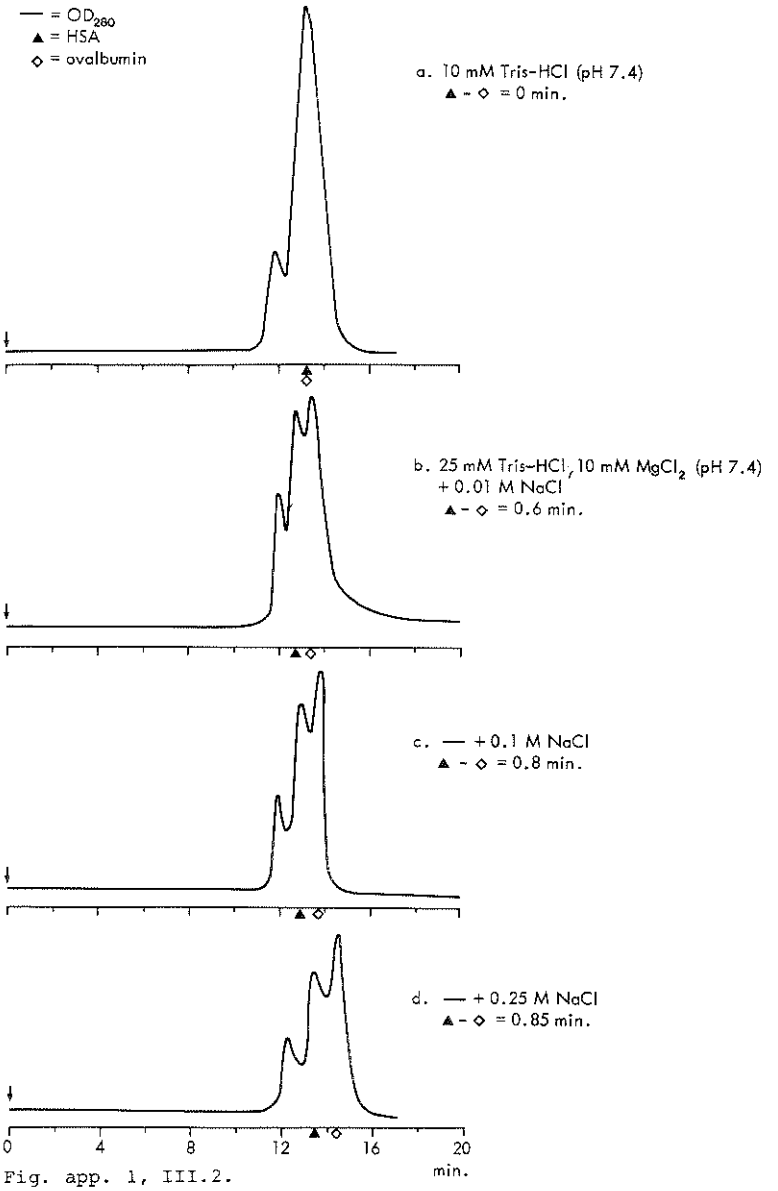


Fig. app. 1, III.2.

The effect of varying the flow rate of the mobile phase (25 mM Tris-HCl, 10 mM $MgCl_2$, pH 7.4) on the retention time of human serum albumin (HSA; MW: 67,000) and ovalbumin (MW: 43,000). 50 µl of a 1 mg/ml solution of each protein was injected simultaneously on a I-125 protein column (Waters Ass., Milford, USA).

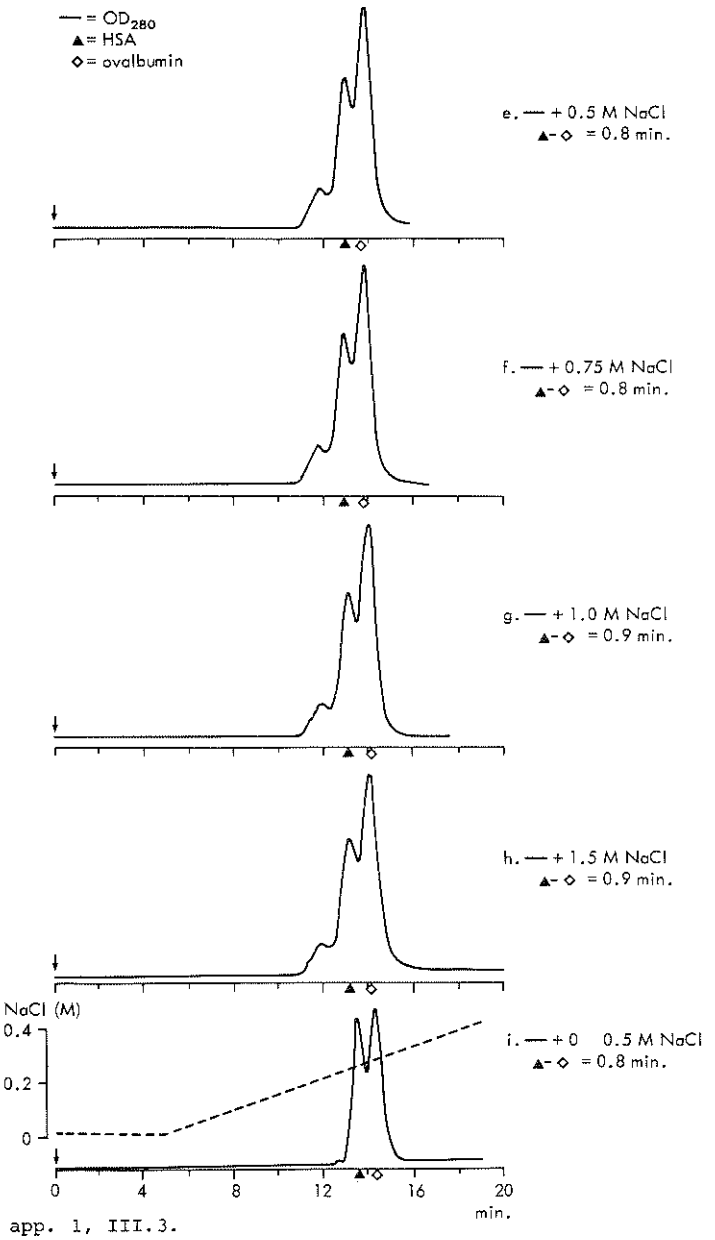
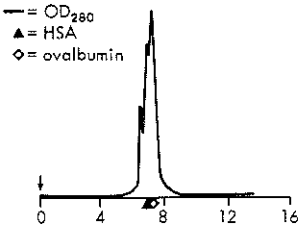
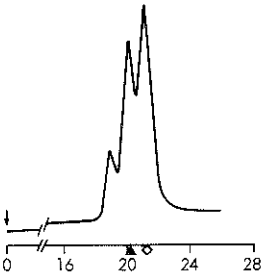


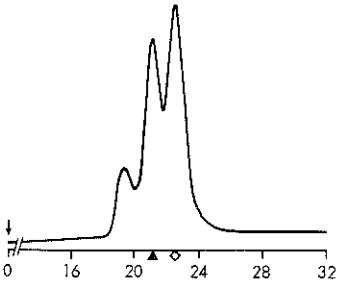
Fig. app. 1, III.3.
The effect of varying the ionic strength of the mobile phase on the retention time of HSA and ovalbumin. Flow rate of mobile phase 0.5 ml/min. Protein concentrations and column conditions as in fig. app. 1, III.2.



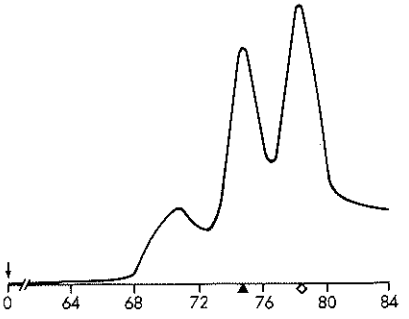
a. flow: 1.0 ml/min.
▲-◇ = 0.3 min.



b. flow: 0.5 ml/min.
▲-◇ = 1.0 min.



c. flow: 0.4 ml/min.
▲-◇ = 1.4 min.



d. flow: 0.2 ml/min.
▲-◇ = 3.6 min.

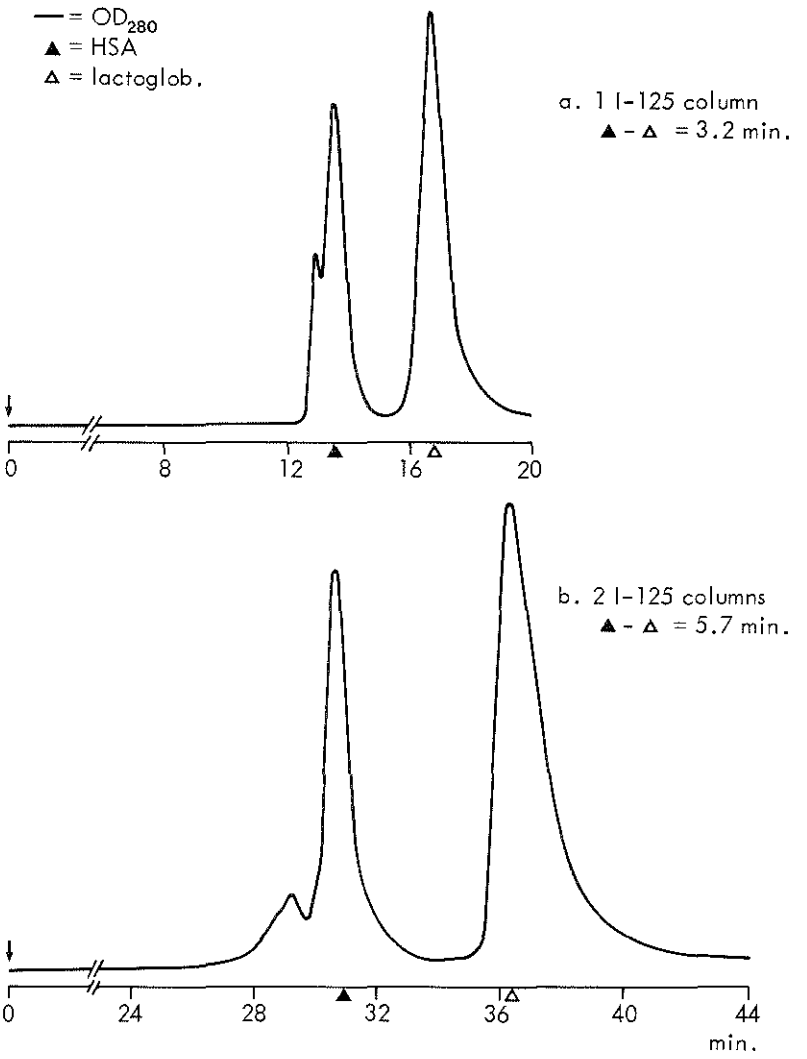


Fig. app. 1, III.4.
The effect of column length on the retention time of HSA (MW: 67,000) and lactoglobulin B (MW: 35,000). Flow rate 0.5 ml/min. Injected sample volume 50 µl (1 mg protein/ml).

App. 1, III.3.1. Influence of flow rate

10 µl of a solution containing HSA (0.5 mg/ml) and ovalbumin (0.5 mg/ml) was injected. The effect of varying the flow rate of the mobile phase (25 mM Tris-HCl, 10 mM $MgCl_2$, pH 7.4) between 0.2-1 ml/min. on the retention time of the proteins is shown in fig. app. 1, III.2.. Separation increased with decreasing flow rate. For practical purposes a flow rate of 0.5 ml/min. was chosen during subsequent testing.

App. 1, III.3.2. Influence of ionic strength

With low ionic strength (10 mM Tris-HCl, pH 7.4) no separation of HSA and ovalbumin occurred (fig. app. 1, III.3.). When the ionic strength was increased either by added NaCl (0.01-1.5 M) or by a gradient (0-0.5 M), the delta (Δ) elution time of the two proteins increased only slightly from 0.6 to 0.9 minutes. Therefore, the addition of NaCl to the mobile phase buffer was concluded to be of no advantage.

App. 1, III.3.3. Influence of column length

As shown in fig. app. 1, III.4., doubling of the retention time of HSA and lactoglobulin occurred when 2 Waters I-125 columns, preceded by a guard column, were applied. The consequent peak broadening and the time increase of the chromatography were quite acceptable.

App. 1, III.3.4. Influence of organic solvent

As AFBP was found to be unstable in various organic solvent solutions (containing methanol, propanol) and these solvents could interfere with the direct measurement and processing of AFBP, no organic solvents were tested.

In all subsequent studies and in the purification of AFBP, 2 protein analysis I-125 columns were used, connected in tandem and preceded by a guard column. The mobile phase (25 mM Tris-HCl, 10 mM $MgCl_2$, pH 7.0) had a flow rate of 0.5 ml/min.

App. 1, III.3.5. Recovery

When partially purified AFBP was applied on two I-125 columns, the recovery of protein was 85-90%.

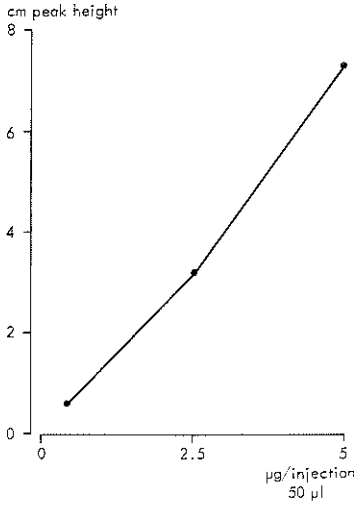


Fig. app. 1, III.5.
Correlation between peak height of OD_{280} extinction and sample protein concentration. HSA dilutions (50 µl aliquots) were injected on one I-125 protein column. Flow rate mobile phase 1 ml/min.

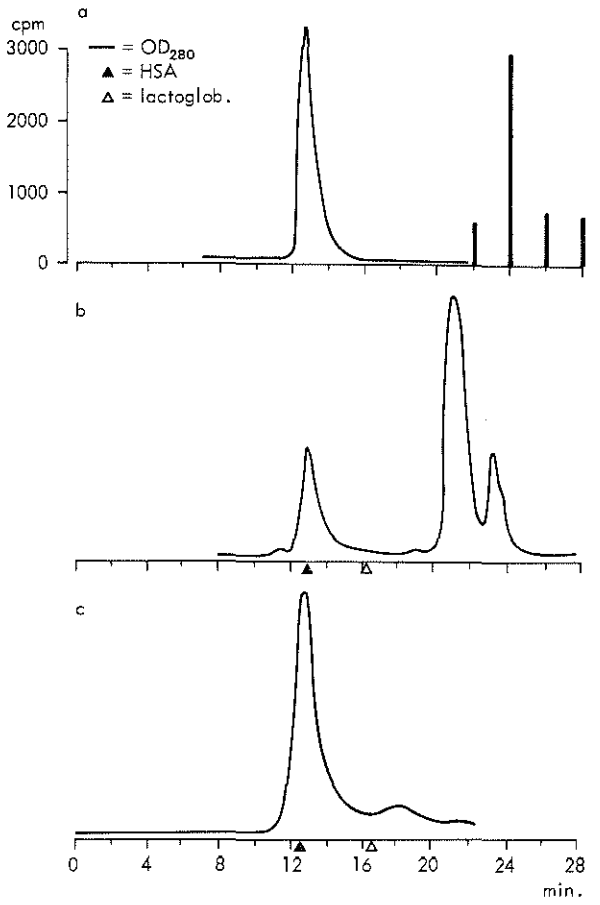


Fig. app. 1, III.6.a.
Determination of void volume (Vo, dextran blue) and total volume (Vt) (Na-I^{125}) of one I-125 protein column. Flow rate 0.5 ml/min. (Tris buffer).

Fig. app. 1, III.6.b.
Elution profile of preterm amniotic fluid. Sample volume 50 µl (protein concentration 3.8 mg/ml) (biorad). Flow rate 0.5 ml/min. (Tris buffer).

Fig. app. 1, III.6.c.
Elution profile of acid-ethanol extract of preterm amniotic fluid (see table III.1.). Sample volume 50 µl (protein concentration 7 mg/ml). Flow rate 0.5 ml/min.

App. 1, III.3.6. Quantitative analysis

As shown in fig. app. 1, III.5., there is good correlation between the maximal extinction of 280 nm and the protein concentration of the applied sample.

App. 1, III.3.7. HSGFC in the purification of AFBP

The effect of high speed gel filtration chromatography of preterm amniotic fluid and acid-ethanol extract is shown in fig. app. 1, III.6. (step 1 and 2, table III.1.).

App. 1, III.4. Discussion

Two modes of interaction between proteins and a protein-separating column, such as the Waters I-125 column, determine the effectiveness of the separation (Majors, 1980; Scott, 1980).
One is the ionic attraction or repulsion of ionizable side chains of the protein and accessible, negatively charged silanol sites on the surface of the silica-based column packing material. Therefore, increasing the ionic strength of the buffer will minimize the electrostatic interaction. An ionic strength of at least 20 mM was found to be necessary to obtain a separation of HSA and ovalbumin (fig. app. 1, III.2.). However, with further increment of the ionic strength, the advantage of improved separation did not outweigh the disadvantage of a high salt concentration in the protein fractions.

The other mode of interaction is the hydrophobic attraction of the alkyl-bonded phase of the silica column to non-polar, aminoacid residues on the surface of the protein molecule. An organic solvent, such as methanol, ethanol or

propanol, can be effective in reducing these interactions, thereby improving the recovery.

As illustrated in table app. 1,III.1., there is quite some variation in regard to the constitution of the mobile phase, its ionic strength, the presence or absence of an organic solvent and the optimal flow rate. With a 20 mM Tris-HCl buffer, HSGFC proved to be a very useful and rapid method for the purification of amniotic fluid binding protein.

As most proteins are sensitive to temperature, it is a disadvantage to perform the HSGFC procedure at room temperature, since it proved to be technically impossible to cool the complete tubing system. The guard column has an important protective function. But even after filtration and chromatography of relatively pure protein solutions, discoloration of the proximal part of the first column occurred and the systemic pressure increased. Furthermore, the gel volume was noted to decline in time, creating a dead space and chromatographic changes. The reason for this phenomenon is not well understood, but it is likely the result of silica being soluble in water and siloxane bonds being labile during basic hydrolysis. "As long as silica based packings are used at high pHs, high buffer strength and at elevated temperatures, shortened column lifetimes will have to be accepted " (Majors, 1980).

Table app. 1, III.1.
Summary of literature data on high speed gel filtration chromatography of proteins.

Author Year	Column type + number	Mobile phase ionic strenght	Organic solvent	pH	Flow rate ml/min.	Recovery %
Barth 1982	Synchropak GPC-100 1	0.05 M Na_2SO_4 1		6.0	0.25	41 - 54
Calam 1981	TSK G 3000 SW 1	0.1 M Na_2PO_4 0.1 M Na Ac		7.0 7.0	0.5	
Gruber 1979	Synchropak GPC 100 1	0.1 M formic acid 50 mM 10% glycerol.phosph.		2.35 7.2	0.06-0.12	± 100
Hefti 1982	Waters I-125 1	0,08 M Na_2HPO_4 + 0,32 M NaCl	20% (v/v) ethanol	7.0	0.1 - 2	78 - 94
Hodge 1979	Waters I-125 3	0,05 M Tris-0,025 M sulfate		7.0	1.0	17 - 86
Jenik 1981	Waters I-125 T-2	0,2 M K_2HPO_4		7.0	1.0	60 - 95
Montelaro 1981	Waters I-125 2 TSK SW 2000 TSK SW 3000	50 mM Na_2HPO_4 or 20 mM Na_2HPO_4 10 mM Na_2HPO_4 + 0,1% SDS	8% guanidine HCl 1% mercapto ethanol	6.5 6.5 6.5	0.2 - 0.4 0.2 - 0.4 0.2 - 0.4	

Table app. 1,III.1. cont.

Author Year	Column type + number	Mobile phase ionic strength	Organic solvent	pH	Flow rate ml/min.	Recovery %
Niemann 1979	Waters I-125 2	0.1 M Na_2HPO_4 + 0.15 M NaCl		7.0	0.5 - 1	± 50
Pfeifer 1982	Waters I-125 2	0.1 M KH_2PO_4 - K_2HPO_4		7.27	1.0	
Rubinstein 1979	Li Chropher diol 1	0.1 M Na Ac	80% n-propanol	7.5	0.25	50 - 100
v. Stetten 1981	Waters I-125 2	0.05 M phosphated NaCl	0,36 M pyridinium formate -n-propanol (75-25)	7.2	1.0	90 - 95
Takagi 1981	TSK G 3000 SW 2	0.1 M Na_2SO_4 0.1% SDS		7.0	0.3	

APPENDIX 2,CHAPTER III

THE EFFECT OF TEMPERATURE AND PH ON THE STABILITY OF AFBP

App. 2, III.1. Introduction

The heat-lability of acid-stable somatomedin inhibitory activity of starved rat serum with a presumed molecular weight of 27-40,000 depends upon pH and the degree of dilution (Salmon et al., 1983).

We investigated the effect of temperature and pH on the stability of amniotic fluid binding protein (AFBP), by determing SM binding capacity and AFBP immunoreactivity in various dilutions of partially purified AFBP. The results indicate that AFBP is heat-stable, irrespective of pH.

App. 2, III.2. Materials, methods and results

Impure AFBP (acid-ethanol extract, step 2, table III.1.) was obtained, as outlined in chapter III, and dissolved in 10 mM Tris-HCl, pH 7.4, in 3 dilutions: 20, 10, and 2 mg/ml (dry weight). All samples were determined in duplicate. Details of the charcoal binding assay (CBA) are described in chapter III. The methodology of the radioimmunoassay (RIA) is given in chapter IV. The correlation between the standards, used in both assays, is given in fig. app. 2, III.1.. Total protein content was determined by the bio-rad method.

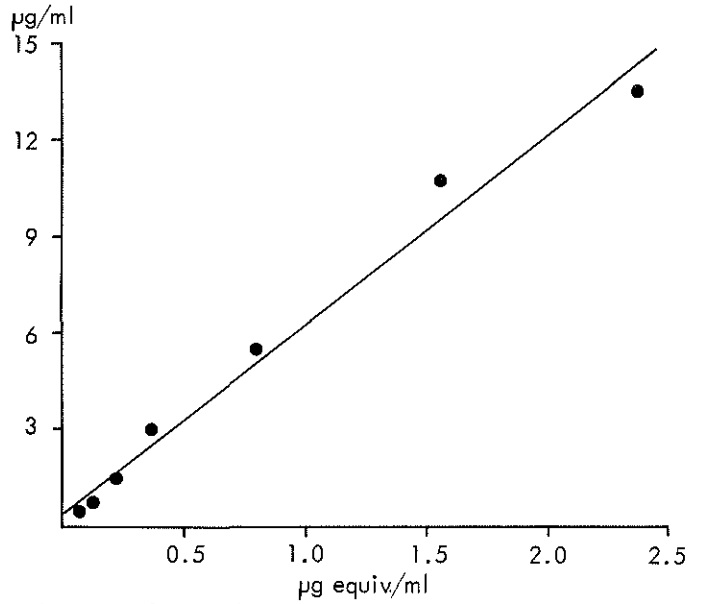


Fig. app. 2, III.1.
Correlation standards used in charcoal binding assay (CBA) and radioimmunoassay (RIA).
Total protein content was measured (Biorad) in dilutions of acid-ethanol extract (step 2, table III.1.), used as standard for the CBA (ordinate), and in dilutions of Sephadex G-150 purified material (step 3, table III.1.), used as standard for the RIA (abscissa).

App. 2, III.2.1. Effect of pH

To 0.1 ml of the AFBP solutions, 0.4 ml Tris-HCl, pH 7.4 buffer (control), 0.1 ml 0.3 M HCl or 0.1 ml 0.3 M NaOH was added. After 3 hours the samples were neutralized with 0.3 M NaOH and 0.3 M HCl respectively at 37°C and assayed. Results are given in fig. app. 2, III.2., indicating no effect of pH on AFBP activity in CBA and RIA.

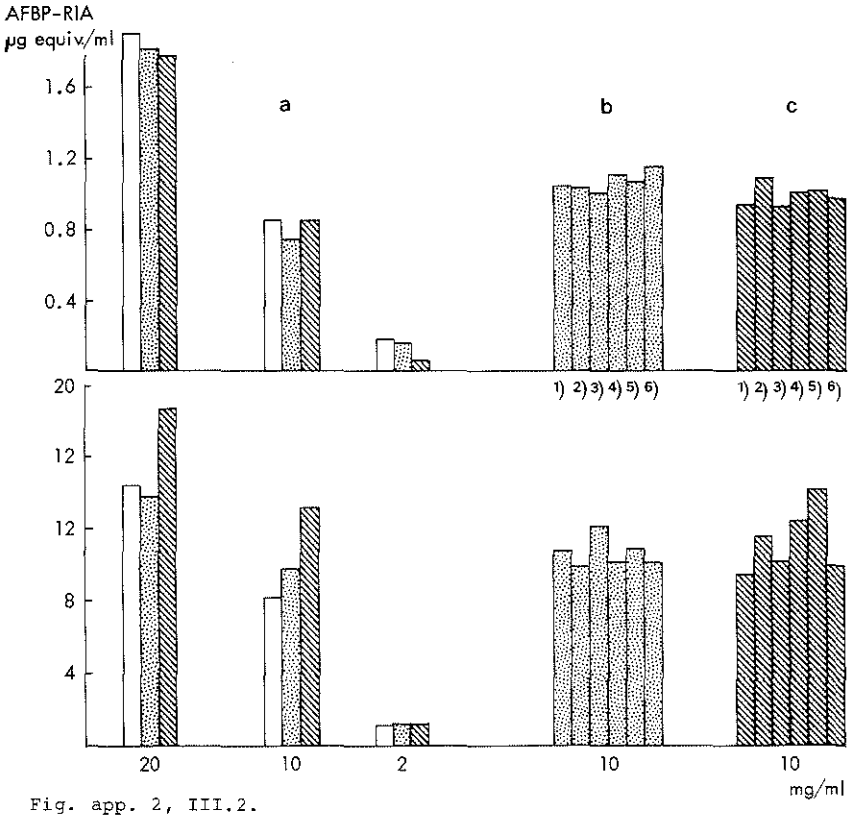


Fig. app. 2, III.2.

Effect of temperature and pH on AFBP.

A. 20, 10, and 2 mg of lyophilized acid-ethanol extract (step 2, table III.1.) were dissolved in Tris-HCl, pH 7.4 buffer, and kept at room temperature for 3 hours (□), at 60°C for 1 hour (▨) and heated at 100°C (▩) for 10 minutes.

B. 10 mg lyophilized acid-ethanol extract (step 2, table III.1.) was dissolved in a buffer, pH range of 2.5-8.5, and heated for 1 hour at 60°C (▨).
1) = pH 8.5 2) = pH 6.5 3) = pH 5.5 4) = pH 4.5 5) = pH 3.5
6) = pH 2.5

C. 10 mg lyophilized acid-ethanol extract (step 2, table III.1.) was dissolved in a buffer, pH range of 2.5-8.5, and heated for 10 minutes at 100°C (▩).
1) - 6) see fig. app. 2, III.2. B.

All fractions were neutralized (pH 7.4) before assaying in AFBP-RIA and AFBP-CBA.

App. 2, III.2.2. Effect of temperature

To 0.1 ml of the AFBP solutions, 0.4 ml of Tris-HCl, pH 7.4 buffer, was added. The tubes were either kept at room temperature for 3 hours or sealed, placed in a water bath at 60°C for 1 hour or at 100°C for 10 minutes. They were centrifuged and the supernatant was assayed. The results are given in fig. app. 2, III.2., indicating no effect of heating at 60°C for 1 hour or boiling at 100°C for 10 minutes on AFBP activity determined by CBA and RIA.

App. 2, III.2.3. Effect of temperature and pH

To 100 µl of the 10 mg/ml AFBP solution, solutions of 0.3 M NaOH and 0.3 M HCl were added, with a pH of 8.5, 6.5, 5.5, 4.5, 3.5 and 2.0 respectively. The tubes were sealed and placed in a water bath at 60°C for 1 hour or at 100°C for 10 minutes. The tubes were centrifuged and the supernatant was assayed. The results are given in fig. app. 2, III.2., and indicate no effect of heating on AFBP activity at a pH range of 2.2 – 8.5.

App. 2, III.3. Discussion

Previously, we reported on the loss of radioreceptorassay activity following heating at 100°C of partially purified AFBP (Drop et al., 1979, chapter II). However, the present study clearly indicates that heating at 60°C for 1 hour or at 100°C for 10 minutes at a range of pH 2.2 – 8.5 does not destroy SM binding capacity or immunoreactivity of AFBP.

The heating appeared to result in slightly higher CBA activity. Evaporation of solvent is an unlikely explanation,

as the tubes were sealed during the heating procedure and centrifuged before assaying. In addition, RIA activity remained unchanged. The heating may have caused an ultrastructural change of the protein, resulting in the exposure of an increased number of binding sites. We conclude that the effect of temperature and pH on AFBP and an SM inhibitory activity of starved rat serum differ only in that, in the latter, activity is lost upon heating at 100°C at pH 5.5.

A practical consequence of this study is that the heat-stability of AFBP allows heating of native serum prior to the determination of AFBP by RIA in native serum. Especially at low dilutions of whole serum, this procedure could lead to an improvement of the sensitivity of the assay, as will be outlined in chapter V.

CHAPTER IV

DEVELOPMENT OF A RADIOIMMUNOASSAY FOR AN SM BINDING PROTEIN IN AMNIOTIC FLUID

IV.1. Introduction

Somatomedin (SM) is a small molecular weight, growth hormone dependent, growth stimulating protein, thought to be involved in the humoral regulation of fetal growth. Evidence stems from the fact that specific receptors for radiolabeled SM have been detected in numerous fetal tissues and that fetal cells produce somatomedins endogeneously (D'Ercole et al., 1980[b]; Hill and Milner, 1981). Although levels of SM activity in cord blood correlate with fetal and early postnatal growth, they generally are discordantly low in comparison with GH levels, which exceed postnatal values (Brinsmead and Liggins, 1978; Sara and Hall, 1980). In amniotic fluid SM is barely detectable by bioassay (Anderson et al., 1974; Bala et al., 1976; Bala et al., 1978; Hill and Milner, 1981).

With an SM radioreceptorassay, a heat-labile, acid-stable SM binding protein was demonstrated (Chochinov et al., 1977; Drop et al., 1979). This binding protein, with a molecular size of $\pm$ 35,000 and pI 4.7, inhibited SM bioactivities in various bioassays (Drop et al., 1979). The levels of this binding protein in amniotic fluid were highest at mid gestation, and their subsequent decrease correlated with fetal renal maturation, suggesting a fetal origin of the binding protein (Chochinov et al., 1976).

In chapter III, we have described an improved procedure for the purification of this amniotic fluid binding protein (AFBP). In this chapter, we report on the development of a specific radioimmunoassay for AFBP (AFBP-RIA).

This AFBP-RIA enabled us to:

a) measure AFBP directly in preterm and term amniotic fluid;
b) demonstrate its presence in pre- and postnatal serum.

IV.2. Materials

Human, bovine and ovine albumin, lactoglobulin A & B, transferrin and ceruloplasmin were purchased from Sigma Chemical Co (St. Louis, Mo.). Alpha-fetoprotein, $alpha_1$-acid glycoprotein, Gc-globulin, $beta_2$-glycoprotein I, alpha-HS-glycoprotein, glycoprotein fraction VI and protein standard plasma* came from Behringer-Calbiochem. (Cal., USA). Sephadex and Sepharose were from Pharmacia Fine Chemicals (Uppsala, Sweden). 99.5% pure Tris was purchased from Koch-Light Laboratories (Colnbrook, U.K.). Dextran-charcoal tablets were provided by Becton & Dickenson (Orangeburg, N.Y.). Anti-human-albumin gamma globulin was obtained from Tago Industries (Burlingane, Cal.), sheep anti-rabbit gamma globulin from Welcome (Beckingham, U.K.).

Preterm amniotic fluid (AF) was obtained for diagnostic purposes by amniocentesis at a gestational age of 16-22 weeks. Term AF was obtained during delivery either per vaginam at the time of rupture of the membranes or during Caesarian section (kindly provided by prof. dr. H.C.S. Wallenburg, department of obstetrics, Erasmus University, Rotterdam).

* The protein standard plasma contained per ml: albumin 38 mg, $alpha_1$-acid-glycoprotein 0.76 mg, $alpha_1$-antitrypsin 2.26 mg, haptoglobulin 1.5 mg, $alpha_2$-macroglobulin 2.2 mg, transferrin 2.77 mg, C_3c 0.79 mg, C_4 0.2 mg, IgG 10.7 mg, IgA 1.63 mg, IgM 1.01 mg (total protein 61.8 mg/ml).

The samples were filtered (paper filter Macherey-Nagel MN 640), pooled and kept frozen at -70°C until processed. Samples showing blood contamination were discarded.

Cord sera were obtained from term and preterm infants (kindly provided by prof. dr. H.C.S. Wallenburg, department of obstetrics, Erasmus University, Rotterdam, and dr. R. Straub, Ikazia Hospital, Rotterdam). Urine was collected from preterm infants. Fetal blood was sampled without contamination by amniotic fluid or maternal blood during a legal abortion. This part of the study was approved by the medical ethics committee of the Sophia Children's Hospital (Erasmus University, Rotterdam). From healthy newborns, children of 1 to 16 years and adults, blood samples were obtained by venipuncture. In all cases, blood was centrifuged at 4°C at 1500 rpm for 20-25 minutes. Serum was collected and kept frozen at -20°C until use.

IV.2.1. Amniotic fluid binding protein (AFBP)

AFBP was isolated from preterm amniotic fluid, as described in chapter III.

IV.3. Methods

IV.3.1. AFBP charcoal binding assay

The methodology of the AFBP charcoal binding assay is explained in chapter III.

IV.3.2. IGF-I/SM-C radioimmunoassay

SM values were determined by IGF-I/SM-C radioimmunoassay (courtesy of dr. H.J. Guyda, McGill University, Montreal), as previously described (Guyda et al., 1981[b]). The antibody to SM-C was obtained through the National Pituitary Agency/ National Institute of Arthritis, Metabolism and Digestive Diseases (USA). IGF-I was iodinated by the chloramine-T method and purified by Sephadex chromatography. Results are normalized against an ORTHO standard serum pool, arbitrarily assigned a value of 1 unit/ml.

IV.3.3. Production of antiserum

Two mature white rabbits, weighing 4 kg each, were immunized. In the primary injection both animals received 150 µg (protein) of HSGFC purified material, dissolved in 250 µl of 20 mM Tris-HCl, pH 7.0, emulsified in complete Freund adjuvant and given subcutaneously at four sites. One rabbit showing detectable AFBP-antibodies was rechallenged at intervals of 4-6 weeks with 150-300 µg (protein) HSGFC purified AFBP, emulsified in incomplete Freud adjuvant. This rabbit was bled from the central ear artery 10 days after each immunization. After clotting, the blood samples were centrifuged at 1500 rpm for 25 minutes at 4°C and the sera were collected and stored at -20°C. Immunogel electrophoresis of this antiserum showed that it was not monospecific and displayed two precipitin lines when reacted against human amniotic fluid. One of the precipitin lines showed a reaction of identity with human albumin. Therefore, rabbit antiserum (3 ml aliquot) was applied to a CN-Br activated Sepharose 4B column (1.6 x 40 cm), to which human albumin (Sigma) was coupled according to the manufacturer's instructions (Pharmacia Fine Chemicals, Uppsala, Sweden).

IV.3.4. Radioimmunoassay of AFBP

HSGFC purified AFBP (10 µg protein) was iodinated by a modification of the chloramine-T method (Drop et al., 1979). The iodination mixture was chromatographed on a Sephadex G-75 column (1 x 25 cm) at 4°C with 0.01 M HCl-0.1% BSA as eluting buffer. Fractions with highest binding to the anti-HSA-free rabbit antiserum (K_{av} 0.1 - 0.25) were pooled and stored at -20°C until use. HSGFC of purified labeled AFBP is shown in fig. IV.1..

IV.3.4.1. Nonequilibrium assay procedure

Reagents were added to 1 x 7.5 cm polyethelene tubes in the following order: to 100 µl of standard or unknown, diluted in 500 µl PBS buffer, was added 100 µl anti-AFBP antiserum (dilution 1:500). After incubation at 4°C for 24 hours, 100 µl ^{125}I-AFBP, about 20,000 cpm, was added. 24 hours later, 100 µl of a 1 : 100 dilution of normal rabbit serum and 100 µl of a 1 : 24 dilution of sheep anti-rabbit antiserum (Welcome Laboratories, Beckingham, U.K.) were added. Following overnight incubation (± 16 hours), the tubes were centrifuged at 3000 rpm for 20 minutes at 4°C. The supernatants were discarded and the pellets counted in a Searle gamma counter (model 1195).

IV.3.4.2. Equilibrium assay procedure

To 100 µl of standard or unknown, diluted in 500 µl PBS buffer, were added 100 µl ^{125}I-AFBP (± 20,000 cpm) and 100 µl anti-AFBP antiserum (dilution 1:500). After incubation for 72 hours, the reaction was terminated by the addition of second antibody and normal rabbit serum as described

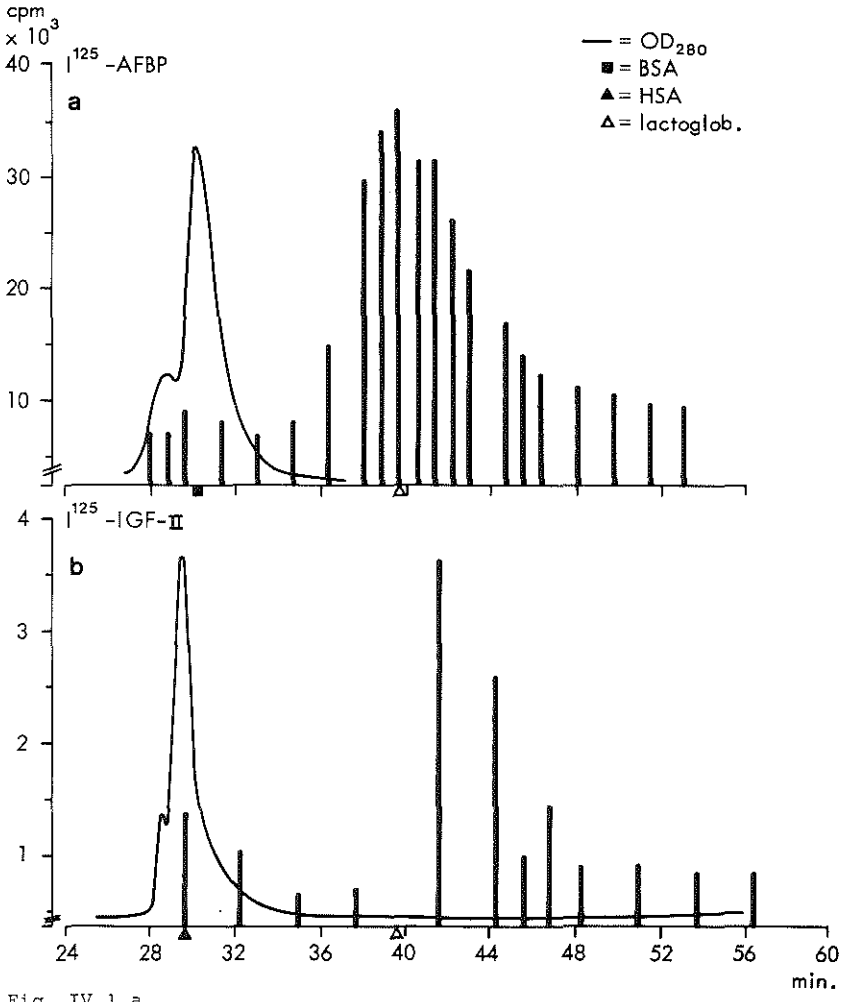


Fig. IV.1.a.
High speed gel filtration chromatography of ^{125}I-AFBP, mobile phase Tris-HCl buffer, pH 7.4. AFBP was iodinated by the chloramine-T method. The iodination mixture was purified by Sephadex G-75 chromatography (buffer: 0.01 M HCl-0.1% BSA).

Fig. IV.1.b.
High speed gel filtration chromatography of ^{125}I-IGF-II (ILAs), running conditions as above. IGF-II was iodinated by the chloramine-T method and purified by adsorption to and elution from a placenta membrane receptor preparation (Posner et al., 1977).

above. It was determined that, at a final dilution of 1 : 5000 of antiserum, 35-40% of ^{125}I-AFBP (± 20,000 cpm, as used in the radioimmunoassay) was bound to antibody in the absence of added unlabeled AFBP. Without the addition of antiserum, 4-6% of ^{125}I was recovered in the pellet, representing a small amount of nonspecific binding of ^{125}I-AFBP in the assay.

IV.3.5. Samples

Amniotic fluid samples and Sephadex G-200 column fractions (see below) were assayed in duplicate at one or more concentrations.

IV.3.6. Gel filtration of serum or plasma under acid conditions

1 ml serum or plasma was acidified with 5 drops of 0.6 M HCl and kept at 4°C for 6 hours. It was then applied on a Sephadex G-200 column (1 x 100 cm), equilibrated in 0.01 M HCl, pH~2. The column was eluted at 4°C with 0.01 M HCl at 0.08 ml/min. The void and inclusion volumes were determined with dextran blue and Na-^{125}I respectively. 2.5-3 ml fractions were lyophilized and reconstituted into 0.4 ml 0.01 M HCl for further analysis.

IV.3.7. Gel filtration of serum or plasma at neutral pH

1 ml of serum or plasma was applied on a Sephadex G-200 column (1 x 100 cm), equilibrated in 0.01 M NH_4HCO_3, pH 7.4. The procedure followed the same steps as described above. Some of the serum samples were acidified with 5 drops of 0.6 M HCl, incubated at 4°C for 6 hours and neutralized

with 5 drops of 0.6 M NaOH, before chromatography at neutral pH.

IV.3.8. Double immunodiffusion in gel

Double immunodiffusion in gel was carried out by the method of Ouchterlony (1962), with gels prepared from 1.5% agar in 0.4 M Tris-barbital buffer, pH 8.8. $AgNO_3$ staining was performed according to the method of Karcher (1980).

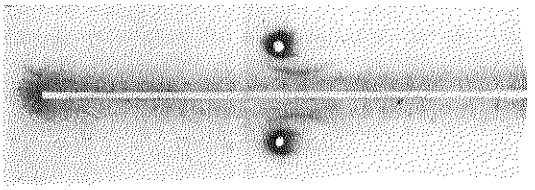


Fig. IV.2.
Immunoelectrophoresis of undiluted anti-HSA-free antiserum and concentrated (10x) preterm amniotic fluid (upper well) and fetal serum (lower well). One precipitin line is visible following silver staining of the gel.

IV.4. Results

IV.4.1. Anti-AFBP antiserum

The antiserum, obtained after immunization of one rabbit with an HSGFC purified AFBP preparation, was not monospecific but showed a second precipitin line following immunoelectrophoresis of preterm amniotic fluid. This contaminating protein was immunologically identified as human albumin. Therefore, the antiserum was freed of anti-albumin by application of the antiserum on an HSA-substituted Sepharose 4B column. Immunoelectrophoresis of concentrated preterm amni-

otic fluid and fetal serum resulted in one precipitin line (fig. IV.2.).

IV.4.2. Radioimmunoassay (characteristics)

Fig. IV.3. shows the displacement of ^{125}I-AFBP from anti-AFBP-antibody by increasing amounts of unlabeled partly purified AFBP during the equilibrium and nonequilibrium assay procedure. The competitive dose-response curve of Sephadex G-150 purified material (step 3, table III.1.), determined by the nonequilibrium assay procedure, was designated as a standard curve and used in all subsequent assays. Results are expressed in µg equiv./ml. Dilution curves of AF, acid-ethanol extract and Sephadex G-150 and HSGFC purified material run parallel to the standard curve (fig. IV.4.). Interassay variation, as determined by assaying the same sample in 14 consecutive assays and expressed as the coefficient of variation, was 10%.

IV.4.3. Hormonal specificity

Hormonal Specificity

A variety of serum proteins listed in table IV.1. was tested for cross-reactivity in the AFBP-RIA. No specific displacement of ^{125}I-AFBP by these proteins was observed at a concentration of 1 mg/ml.

IV.4.4. Binding studies AFBP-RIA

The bound/free ratio was plotted against the bound fractions of dilutions of partially purified AFBP (Sephadex G-150 purified material (step 3, table III.1.)). The data suggest the presence of at least two classes of binding sites of the antibody (fig. IV.5.).

IV.4.5. Amniotic fluid samples

AFBP levels in preterm and term AF determined by AFBP-RIA are given in fig. IV.6.. The mean ± SEM AFBP level in 30 preterm AF samples (gestational age of 16-20 weeks) was

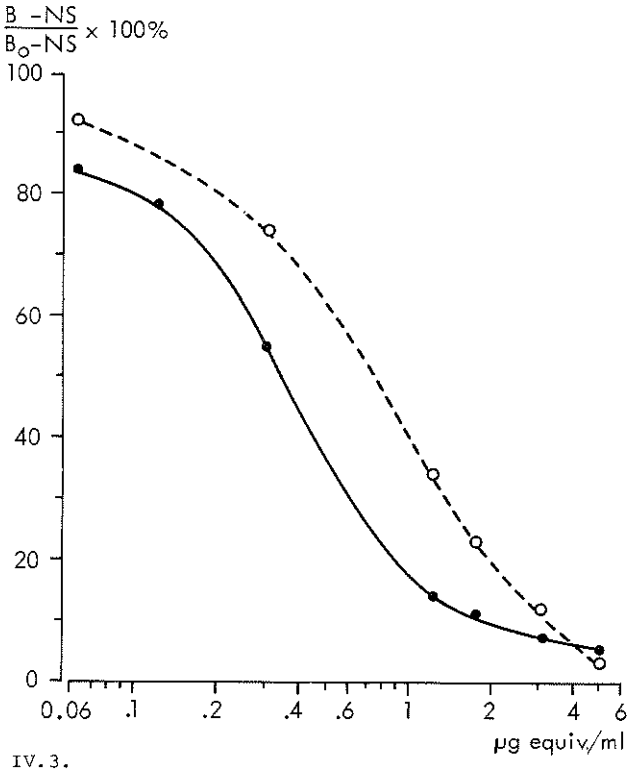


Fig. IV.3.
AFBP Radioimmunoassay.
Competitive-dose response curve for a partially purified AFBP preparation (step 3, table III.1.). AFBP content is expressed as µg equiv. (protein)/ml and plotted against 100 x $\frac{B - NS}{Bo - NS}$, where Bo = ^{125}I-AFBP, bound in the absence of added unlabeled AFBP, and B = ^{125}I-AFBP, bound in the presence of different concentrations of unlabeled hormone.
o-----o = equilibrium assay procedure; ●——● = non-equilibrium assay procedure.

148 ± 18 µg equiv./ml. In term AF (38-40 weeks of gestation) the mean (± SEM) value was 72 ± 11 µg equiv./ml (n = 12). The difference between the mean preterm and term values is statistically significant ($p < 0.05$). IGF-I/SM-C values (± SEM) in preterm and term amniotic fluid were uniformly very low (mean 0.08 ± 0.01 U/ml and 0.08 ± 0.02 U/ml respectively; no statistical difference).

Table IV.1.

Cross-reactivity (concentration 1 mg/ml)

Protein	MW	$\frac{B-NS}{B_O-NS}$ x 100%
Lactoglobulin A	35,000	97
Lactoglobulin B	35,000	100
β_2-glycoprotein	40,000	97
α_2-acid-glycoprotein	40,000	99
Ovalbumin	43,000	99
α_2-HS-glycoprotein	49,000	90
Gc-globulin	50,000	97
α-fetoprotein*	64,000	94
BSA	67,000	99
HSA	68,000	94
Transferrin	75,000	96
Ceruloplasmin	13,200	97
Glyco-protein fraction IV (Behringer)	-	95
Normal standard serum (Hoechst)	-	100
ILAs (IGF-II)**	9,400	98

*0.2 µg/ml

**25 ng equiv./ml

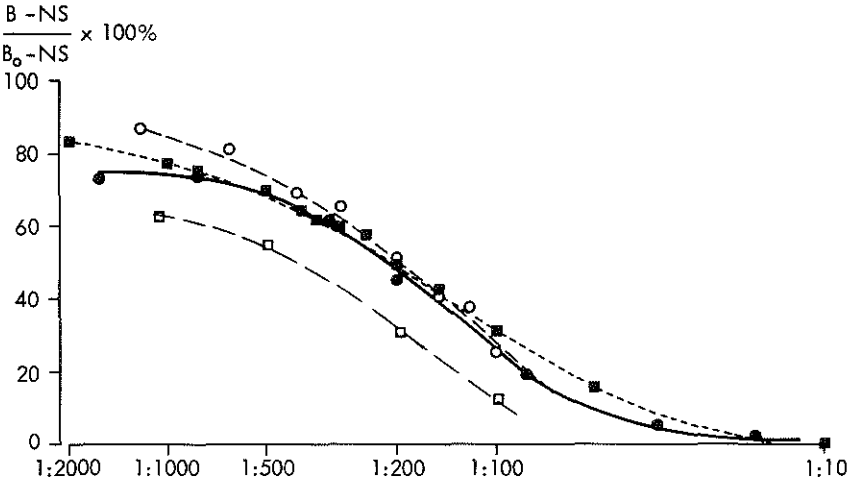


Fig. IV.4.
Dilution curves of preterm amniotic fluid and partially purified AFBP showing parallelism.
●——● standard; □-—□ amniotic fluid; ○-·-○ ethanol-extract;
■·····■ HSGFC purified AFBP.

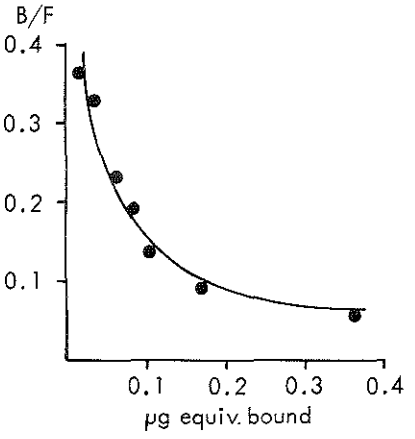


Fig. IV.5.
Scatchard analysis of the AFBP-RIA standard curve obtained by non-equilibrium assay procedure, suggesting the presence of at least two classes of binding sites of the rabbit antibody.

IV.4.6. Urine samples

In 3 urine samples of preterm born infants (gestational age 32-34 weeks), AFBP values were 0.08, 0.08 and 0.1 µg equiv./ml.

IV.4.7. Gel filtration of amniotic fluid and serum

Preterm AF was chromatographed on Sephadex G-150 columns, at both pH~2 and 7.4. AFBP-RIA activity was eluted at K_{av} 0.45-0.6 (fig. IV.7.), similar to AFBP-CBA activity (cf. fig. III.2.).

When serum was chromatographed on Sephadex G-200 at pH 2.2, AFBP-RIA activity was eluted in one peak at K_{av} 0.6. Highest activity was found in fetal serum (gestational age of 20-22 weeks) (fig. IV.8.a.), lowest in serum from adults (male or female) (fig. IV.8.c., d.). It was possible to demonstrate AFBP-RIA activity in serum of GH-deficient patients (fig. IV.8.e.). No activity was present in rat, rabbit, porcine or bovine serum (data not shown).

The effect of pH and prior acidification (see Methods for details) on the elution profile of fetal and cord serum is shown in fig. IV.9. and fig. IV.10.. When fetal serum was chromatographed at neutral pH, the predominant form of SM binding activity was found at K_{av} 0.5-0.75, corresponding with a molecular weight range of 30-60,000 (fig. IV.9.a.). IGF-I/SM-C RIA activity was found in the corresponding fractions (fig. IV.9.c.). Under acid conditions, however, SM binding activity, as determined by AFBP-CBA activity, was found in the large molecular weight range at K_{av} 0.1-0.4 (fig. IV.9.a.). AFBP-RIA activity was found only at K_{av} 0.5-0.75, irrespective of pH or prior-acidification of both fetal and cord blood. Fractions

were generally higher when serum had been acidified.

When fetal serum, previously acid-treated as outlined above, was chromatographed at neutral pH in the presence of labeled SM (IGF-II), radioactivity was still eluted at K_{av} 0.6 only (data not shown).

Large and small molecular weight SM binding proteins were present in cord blood (fig. IV.10.a.b.c.). At neutral pH, IGF-I/SM-C predominantly eluted at K_{av} 0.15-0.4, corresponding with a molecular weight range of 150-200,000.

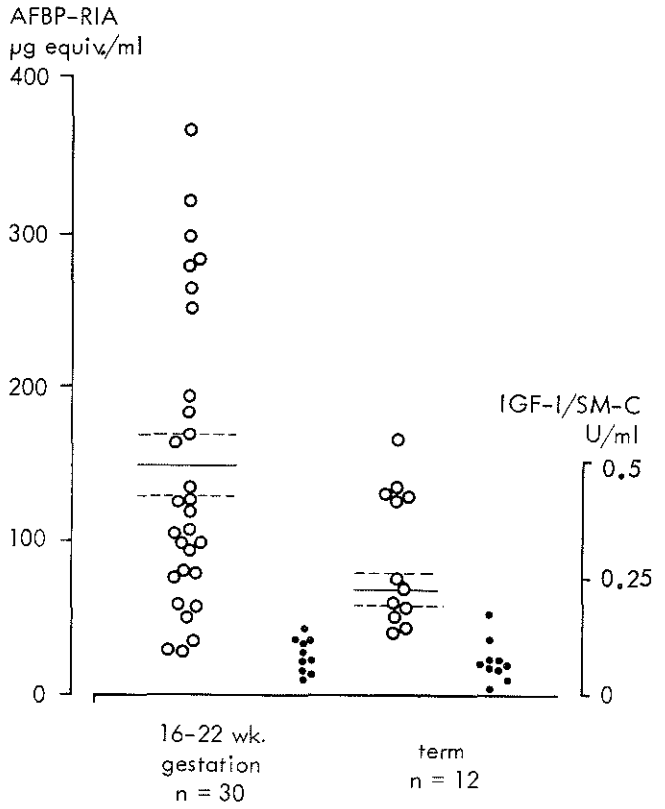


Fig. IV.6.
Levels of AFBP (O), measured by RIA (ordinate, left), of 30 unselected preterm AF samples (gestation of 16-22 weeks) and 12 term AF samples. SM values (●) were determined by IGF-I/SM-C RIA (ordinate, right).
—— mean ---- SEM.

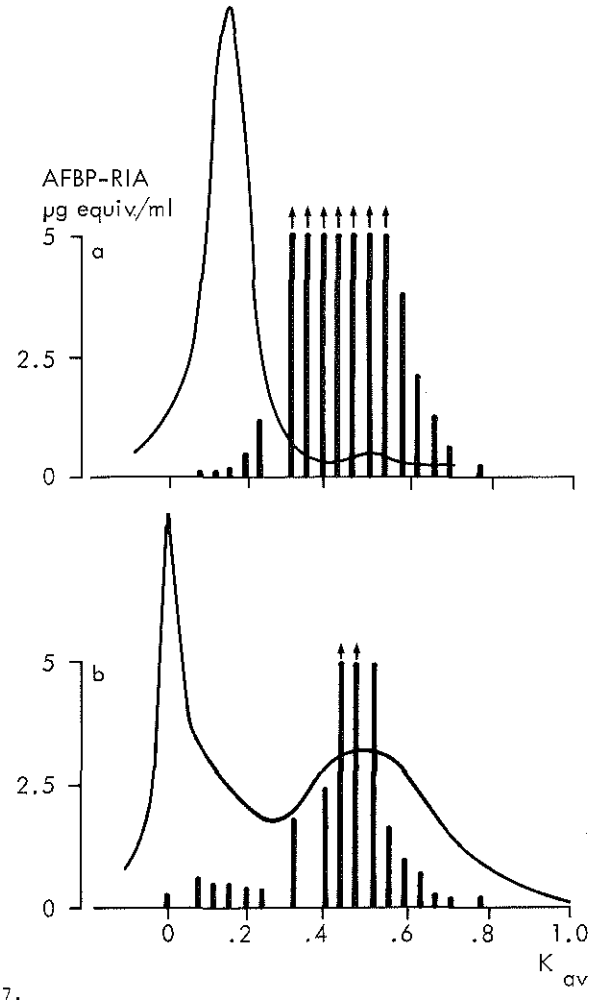


Fig. IV.7.

Sephadex G-150 chromatography (column size 1 x 100 cm) of preterm amniotic fluid (fig. IV.7.a.) and ethanol-extract (fig. IV.7.b.). Buffer: 0.01 M HCl, pH ~ 2. 1 ml fractions were lyophilized and reconstituted into 0.2 ml assay buffer. AFBP-RIA activity was recovered only in fractions at K_{av} 0.45-0.6, similar to AFBP-CBA activity (cf. fig. III.2.) and similar to the elution volume of labeled AFBP. The elution profile of AF chromatographed at neutral pH (Tris-HCl, pH 7.4) was identical (data not shown).

~ = OD_{280}; ▮ = AFBP-RIA activity.

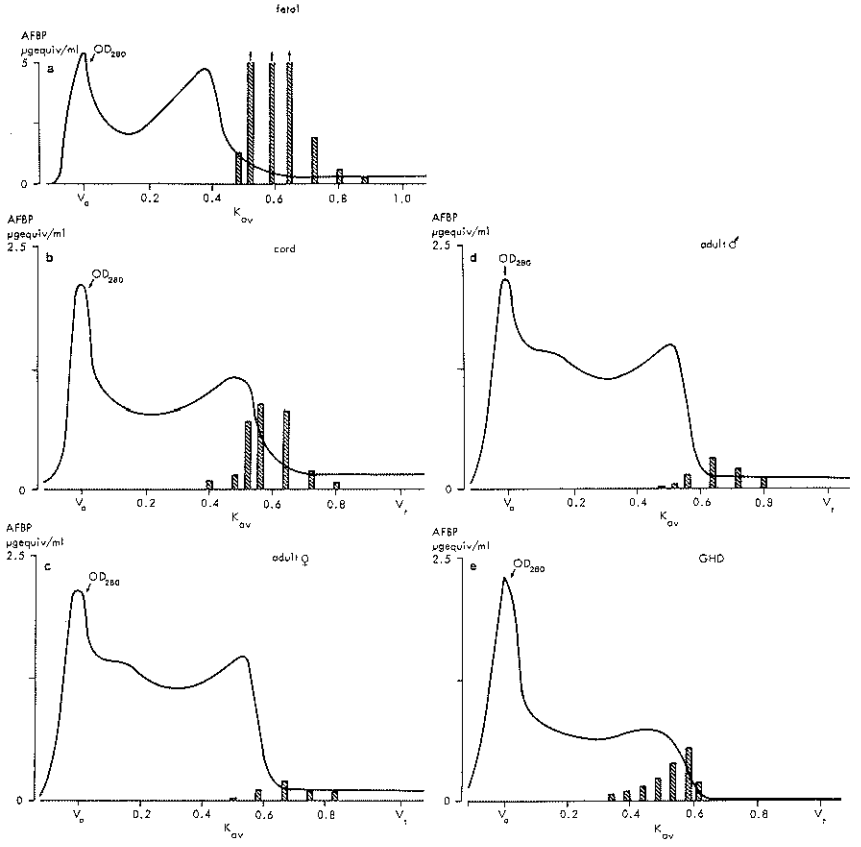


Fig. IV.8.
Sephadex G-200 chromatography (column size 1 x 100 cm) of 1 ml serum. Buffer: 0.01 M HCl, pH ∼ 2. 1 ml fractions were lyophilized and reconstituted into 0.2 ml assay buffer.
a) fetal (± 20 weeks of gestation); b) cord (term); c) adult (female); d) adult (male); e) patient with GH deficiency (male, 7 yrs.).

IV.5. Discussion

In this chapter, the development of a specific radioimmunoassay for AFBP is described. The antiserum was prepared by injection of HSGFC purified AFBP into two rabbits. Only one rabbit responded with an increase of antibody titer. The antiserum was cleared of anti-albumin antibodies by application of the antiserum over an HSA-substituted Sepharose 4B column. That the antibody is directed against AFBP is suggested by its binding to chromatographically purified I^{125}-AFBP. AFBP-RIA activity was demonstrated in polyacrylamide disc-gel fractions showing AFBP-CBA activity (see fig. III.4.). The protein isolated from these fractions stained as a single band, following analytical polyacrylamide gel electrophoresis (see fig. III.6.). Monospecificity is suggested by the lack of cross-reactivity of a limited number of proteins tested (table IV.1.) and by the result of immunoelectrophoresis of concentrated preterm amniotic fluid and fetal serum in the presence of antiserum (fig. IV.2.). The sensitivity of the assay was improved by adoption of the nonequilibrium procedure, as shown in fig. IV.3..

SM bioactivity has been demonstrated in amniotic fluid (Anderson et al., 1974; Bala et al., 1976, 1978; Hill and Milner, 1981). Generally values were very low, slightly higher at term than in early pregnancy (Bala, 1978). It was previously reported that no small molecular weight insulin-like activity (ILAs) was detectable by placenta membrane radioreceptorassay in (pre)term AF, even under rigorous protein dissociating conditions, such as acid-ethanol extraction and chromatography in 8 M urea (Drop et al., 1979). Our present results indicate that IGF-I/SM-C radioimmunoassay activity is barely detectable in preterm and term AF.

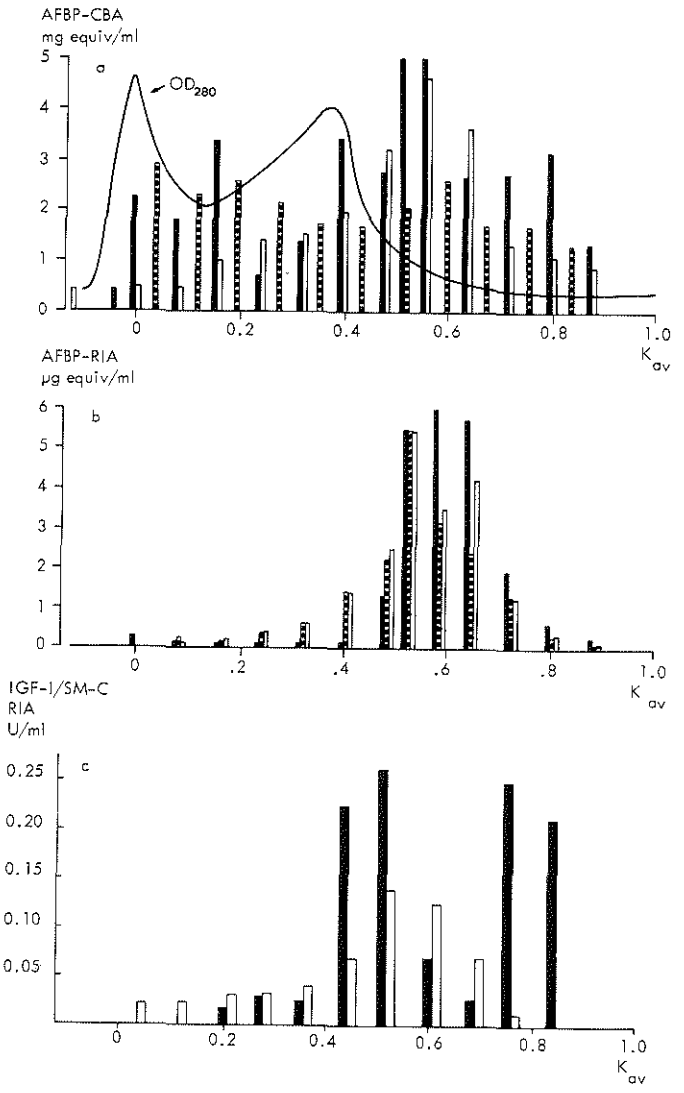

AFBP-CBA
mg equiv/ml
a
OD280
AFBP-RIA
µg equiv/ml
b
IGF-I/SM-C
RIA
U/ml
c
Kav

Fig. IV.9.
Sephadex G-200 chromatography (column size 1 x 100 cm) of fetal serum.

■ 1 ml serum was acidified with 5 drops of 0.6 M HCl, kept at 4°C for 6 hours and chromatographed in 0.01 M HCl, pH 2.2.

□ 1 ml serum was chromatographed in 0.01 M NH_4HCO_3, pH 7.4.

▤ 1 ml serum was acidified with 5 drops of 0.6 M HCl, kept at 4°C for 6 hours, neutralized with 5 drops of 0.6 M NaOH and chromatographed in 0.01 M NH_4HCO_3, pH 7.4.

The fractions were lyophilized and reconstituted with assay buffer into 1/5 of the original volume.

a. The fractions were measured by AFBP-CBA. At neutral pH the predominant activity is found at K_{av} 0.45-0.7. Following acidification of the serum, some of the SM binding is retained in the large molecular weight range.

b. The fractions were measured by AFBP-RIA. There is only assayable activity at the elution volume of labeled AFBP (K_{av} 0.5-0.7). The AFBP-RIA activity in the fractions of acidified serum appears to be higher than in the fractions of serum chromatographed at neutral pH.

c. IGF-I/SM-C content of the fractions was measured by RIA. At neutral pH predominant elution of activity occurred at K_{av} 0.45-0.7. After acidification, unbound SM is found at K_{av} 0.8.

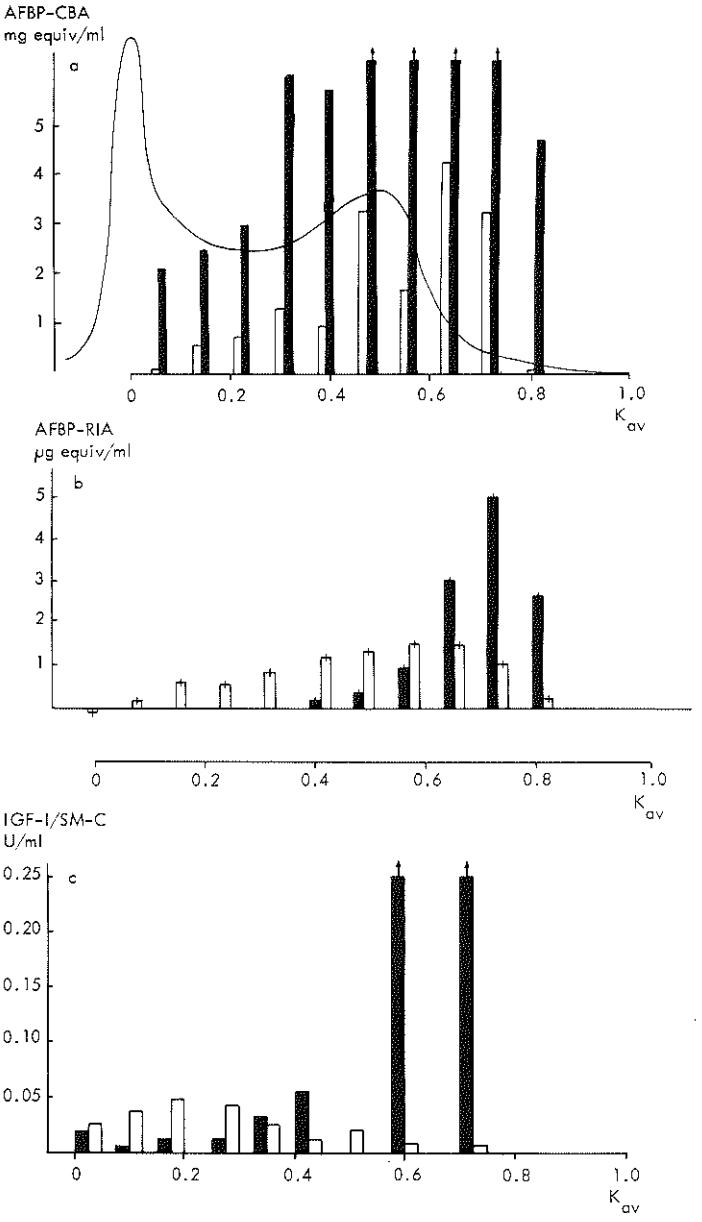

AFBP-CBA
mg equiv/ml
a
5
4
3
2
1
0
0.2
0.4
0.6
0.8
1.0
K_{av}
AFBP-RIA
µg equiv/ml
b
5
4
3
2
1
0
0.2
0.4
0.6
0.8
1.0
K_{av}
IGF-I/SM-C
U/ml
c
0.25
0.20
0.15
0.10
0.05
0
0.2
0.4
0.6
0.8
1.0
K_{av}

Fig. IV.10.

Sephadex G-200 chromatography (column size 1 x 100 cm) of cord serum.

■ 1 ml serum was acidified with 5 drops of 0.6 M HCl, kept at 4°C for 6 hours and chromatographed in 0.01 M HCl, pH 2.2.

□ 1 ml serum was chromatographed in 0.01 M NH_4HCO_3, pH 7.4.

a. The fractions were measured by AFBP-CBA. At neutral pH activity is found not only at K_{av} 0.45-0.7, but also at K_{av} 0.2-0.4. Following acidification, the activity was markedly increased.

b. The fractions were measured by AFBP-RIA. Findings similar to fig. IV.9.b.

c. The fractions were measured by IGF-I/SM-C RIA. At neutral pH predominant elution of activity at K_{av} 0.15-0.4. After acidification, activity shifts to K_{av} 0.6-0.8, representing unbound SM.

Chochinov's suggestion, that SM binding activity in AF is of fetal origin (Chochinov et al., 1976), may well be correct since AFBP-RIA activity was found in urine of preterm infants.

The chromatographic profile of preterm amniotic fluid and pre- and postnatal serum is identical, pointing to a protein (or proteins) with an apparent molecular weight of ± 40,000. No RIA activity could be demonstrated in the large molecular weight range at neutral pH or under acid conditions. As shown in fig. IV.8., 9. and 10., it is evident that AFBP-RIA activity is present in pre- and postnatal serum. Activity appears to be highest in fetal serum, lowest in adult serum.

We confirm the observation by D'Ercole (1980) that at neutral pH the elution patterns of IGF-I/SM-C in fetal and cord serum differ. In fetal serum of infants of less than 27 weeks of gestation, IGF-I/SM-C elutes at an apparent molecular weight of 40,000 (fig. IV.9.c.), whereas in cord serum the predominant elution occurs at a molecular weight of 150-200,000 (fig. IV.10.c.). SM binding activity, as determined by the AFBP-CBA, is found in the corresponding fractions (fig. IV.9.a., 10.a.). When fetal serum, however, is acidified and chromatographed at either acid or neutral pH, SM binding activity is also discovered in the large molecular weight range. By acidification of serum, SM is separated from its binding proteins, and the binding sites of the binding proteins are increased (Daughaday et al., 1980). In fetal serum, large molecular weight binding proteins may be present. Because of the low content of IGF-I/SM-C in fetal serum, they may escape detection when IGF-I/SM-C is measured in the individual fractions (fig. IV.9.c.). Alternatively and more likely, the AFBP-CBA activity found at K_{av} 0.1-0.4 may represent the elution of small molecular weight binding proteins, nonspecifically bound to large molecules.

Sara measured SM activity in human serum by radioreceptorassay, utilizing human fetal brain plasma membrane as matrix (Sara et al., 1981). Levels in fetal serum were found to be increased, not only compared with adult values, but also with SM values measured by radioimmunoassay. On the basis of this discrepancy between SM values measured in fetal serum by RRA and RIA, Sara postulated that in the human fetus an embryonic form of SM exists. At the end of the first half of gestation, this embryonic SM is present at high concentrations. During the second half of gestation, the values gradually fall as adult forms of SM begin being produced. However, in this study native serum was tested and fetal serum was not subjected to another RRA. This chapter describes that substantial quantities of SM binding activity in fetal serum have been demonstrated. Therefore, the discrepancy between RRA and RIA SM values in fetal serum can be fully explained by interference of SM binding proteins, in the RRA, such as AFBP, leading to spuriously high values.

The next step in the investigation of the biological significance of AFBP, now shown to be present in pre- and postnatal serum, was to explore its relationship with small molecular weight SM in pre- and postnatal serum and in serum of patients with various growth disorders. In chapter V, results of some initial studies are presented.

CHAPTER V

AFBP DETERMINATION IN NATIVE SERUM OR PLASMA

V.1. Introduction

It has been well established that small molecular weight SM circulates in plasma, mainly bound to macromolecular carrier or binding proteins (a.o. Zapf et al., 1975; Hintz and Liu, 1977; Furlanetto, 1980; Daughaday et al., 1982[a]). At least two different binding proteins with high affinity and specificity for SM exist. The major one (150-200,000 MW) consists of two protein subunits, one of which is acid-stable and contains the structural determinants necessary for binding SM, while the other is acid-labile (Furlanetto, 1980). The acid-stable subunit appears to be under GH control (a.o. Zapf et al., 1978; Copeland et al., 1980; White et al., 1981; Schalch et al., 1982). In addition, plasma contains an unsaturated, acid-stable form of an SM binding protein with a 35-50,000 MW (Hintz et al., 1981). Thusfar, plasma SM binding activity has been determined by incubation of serum fractions with labeled SM and separation of unbound from bound SM with dextran-charcoal (Moses et al., 1979; Zapf et al., 1980; Hintz et al., 1981; Daughaday et al., 1982[a]).

The biological significance of the serum concentration of this SMBP is largely unknown. Evidence reveals that under physiological and various pathological conditions the serum SM binding activity differs greatly. Levels of the unsaturated SMBP ($\pm$ 40-50,000 MW) are elevated in serum of patients with GH deficiency and decline during GH treatment (Hintz et al., 1981). Increased values have been

found in cord serum and in serum of uremic patients (Borsi et al., 1981; Goldberg et al., 1982). The SM binding proteins are probably generated in the liver (Binoux et al., 1982).

The availability of a specific RIA for a ± 40,000 MW SM binding protein, isolated from preterm amniotic fluid, allowed an initial exploration of the relationship between AFBP and small molecular weight SM in pre- and postnatal serum and of its homology with known serum SM binding proteins.

V.2. Materials and methods

V.2.1. Samples

Human fetal and cord blood was obtained as outlined in chapter IV. From newborns, children and adults, blood samples were obtained by venipuncture between 9 and 11 am. In all cases, blood was centrifuged at 4°C at 1500 rpm for 20-25 minutes. Serum or plasma was collected and kept frozen at -20°C until use.

V.2.2. AFBP-RIA

AFBP was assayed in native serum or plasma (dilution range 1:2 / 1:600) by radioimmunoassay as outlined in chapter IV, with the following modification: to the tubes containing the standard series (dilutions of Sephadex G-150 purified acid-ethanol extract from preterm amniotic fluid (step 3, table III.1.)), 100 µl of a 1% solution of bovine serum was added. All serum values are the mean of the two highest dilutions fitting the standard curve. It was established that AFBP-RIA values of native serum and plasma give similar results.

V.2.3. SM determinations

Total immunoreactive SM was determined by RIA (Bala and Bhaumick, 1979). IGF-I/SM-C was measured by RIA (Guyda et al., 1981), and IGF-II was measured by RRA (Posner et al., 1977). Results are expressed in Ortho standard serum units per ml.

V.2.4. Conditions studied

The following conditions were studied:

V.2.4.a. Diurnal variation

Plasma samples were obtained from 8 adult volunteers (4 male, 4 female; age range of 28-42 years), at 0800-0900 h, 1300-1400 h and 2200-2300 h on one day. In addition, from a 12.5-year old girl (after the recent onset of insulin-dependent diabetes mellitus) and from a 10-year old girl with hypopituitarism, plasma samples obtained throughout a 24-hour period were assayed. Glucose, growth hormone and cortisol determinations were performed according to established methods.

V.2.4.b. Age dependence

AFBP values were measured in fetal serum (estimated gestation of 18-22 weeks), cord serum (32-40 weeks of gestation) and in serum or plasma from children (boys and girls) of 1-17 years of age and healthy adults (hospital and laboratory personnel). The children had been referred to the pediatric outpatient clinic of the Sophia Chil-

dren's Hospital, Rotterdam, for minor illnesses. Patients with endocrine disorders and liver/renal failure were excluded (see below).

V.2.4.c. GH dependence

a. From 14 patients with GH deficiency, age range of 0.5-15 years, plasma was obtained before GH treatment was initiated. The diagnosis of GH deficiency was based on short stature (standard deviation score corrected for height (SDS_h) less than -2.3), delayed bone maturation and an insufficient GH response during at least two stimulatory tests, while the patient was euthyroid. In 3 patients on GH treatment (5 mg Nanormon ^{R} i.m. twice weekly for 9-27 months), the effect of a single GH injection was investigated.

b. HGH was administered to 6 patients with GH deficiency (3 male, 3 female, age: 8-16 years). All subjects conformed to the criteria for GH deficiency, established in Canada. The peak serum GH level after insulin-induced hypoglycemia and arginine infusion was less than 5 ng/ml. Fasting plasma samples were obtained at 0, 4, 24 and 72 hours after a single injection of HGH and after 1 and 6 months of chronic HGH therapy. Results of this study have been reported previously (Guyda et al., 1981[b]).

c. From a patient with Laron dwarfism (age: 22 years), blood was obtained. This patient has been described in detail (Van den Brande et al., 1974[b]).

d. Blood was obtained from a girl (age: 14 years) with growth acceleration and biochemical evidence of acromegaly.

V.2.4.d. Sex-steroid dependence

From adolescents with tall stature (15 girls and 9 boys; diagnosis: constitutional tall stature in 22 patients, Marfan Syndrome in 2 patients; age: 10-15 years), plasma samples were obtained before and during treatment (2-8 months) with high doses of estrogens in girls (ethynylestradiol 200 µg/d p.o.) and androgens in boys (Sustanon ^{R}, Organon, 250 mg i.m. once weekly). Details about this study will be reported on separately (Hokken-Koelega et al., in preparation). SM-C and AFBP levels were measured in the same samples.

V.2.4.e. Other physiological and pathological conditions

From 6 patients (4 boys, 2 girls; age: 5-18 years) with end stage renal failure and on hemodialysis treatment, serum was obtained prior to a dialysis session. Serum (1 ml) of 3 patients was chromatograpned on a 1 x 100 cm Sephadex G-200 column at neutral pH. From healthy pregnant women attending the obstetric outpatient clinic (head prof. dr. H.S.C. Wallenburg) of the University Hospital Dijkzigt, Rotterdam, blood was obtained at 36 weeks of gestation.

V.2.4.f. Other species

Blood was obtained from nonimmunized rats, rabbits, orang-outangs (mothers and children, age: 7-18 months) and an elephant (courtesy of dr. M. Frankenhuis V.D., Blijdorp Zoo, Rotterdam). Bovine and porcine blood was obtained from a local slaughterhouse.

V.2.4.g. In vitro studies

The supernatant of placenta cell explants, obtained from a preterm placenta (gestation of 17½ weeks) and from a term placenta (courtesy of Wei Lai M.Sc., McGill University, Montreal), was assayed for AFBP-RIA activity, following 1-5 days of incubation in a culture medium containing 10% fetal calf serum. The supernatant of a hepatoma cell line (PLC/PRF/5; MacNab et al., 1976) was similarly assayed (courtesy of dr. H.K. Yap, department of internal medicine, Radboud Ziekenhuis, Nijmegen).

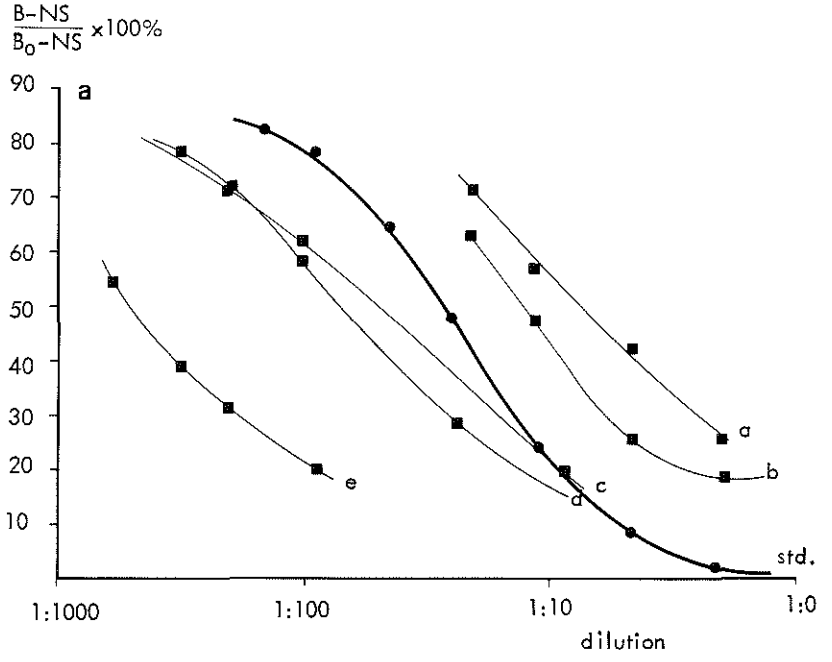


Fig. V.1.a.
AFBP-RIA activity of dilutions of native fetal serum samples (a-e) as compared to the standard series (std) (dilutions of Sephadex G-150 purified acid-ethanol extract of preterm amniotic fluid, step 3, table III.1), to which a 1% solution of bovine serum has been added.

V.2.5. Statistical analysis

Where appropriate, results are expressed as means ± standard error of the mean; the data were analysed on a Hewlett packard desk top computer type 9825B. Significance was determined by the Krustal-Wallis test, as described by Conover (1980).

V.3. Results

V.3.1. AFBP-RIA of native serum/plasma

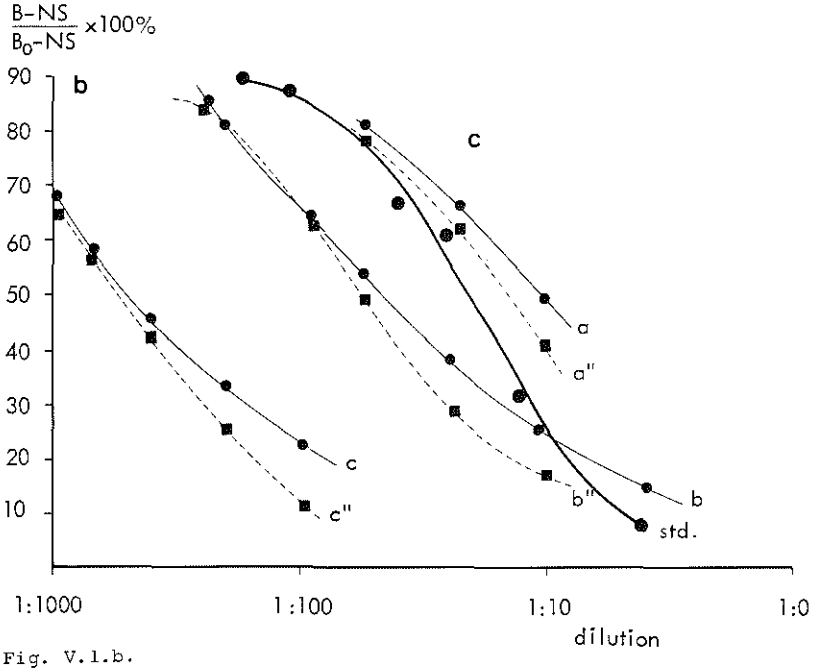


Fig. V.1.b.
AFBP-RIA activity of dilutions of fetal serum samples (untreated a, b, c ——— ; acid-treated a', b', c' ----), as compared to the standard series (std).
Acid treatment of serum results in a slight shift to the left of the dilution curve.

Serum dilutions in a range of 1:20 / 1:600 generally showed parallelism with the standard curve (fig. V.1.a.). In serum diluted less than 1:20, the difference between the mean value and the two values, from which the mean was calculated, averaged 38% of the mean value (range 10-65%; n = 19). Prior acidification of the serum or plasma sample with 0.6 M HCl for 4-6 hours at 4°C, followed by neutralization with 0.6 M NaOH, resulted in a slight shift of the dilution curve "to the left" (fig. V.1.b.). The calculated AFBP values were generally higher than those in untreated serum or plasma.

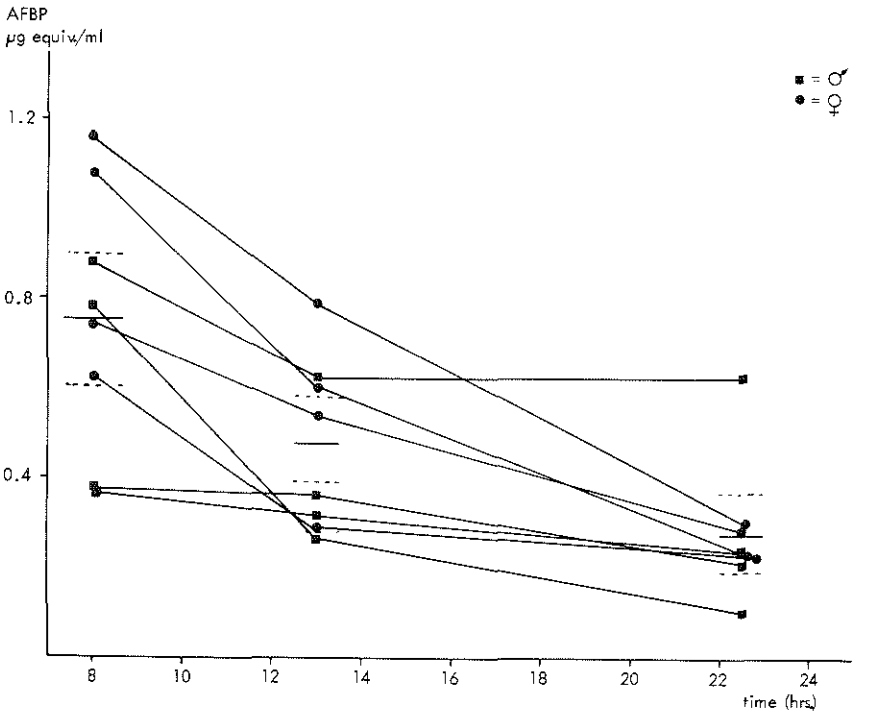


Fig. V.2.
Diurnal variation of AFBP plasma values in 8 adult volunteers (4 males, 4 females). The mean morning value (± SEM) was 0.7 ± 0.1 µg equiv./ml and differed significantly from the mean afternoon 0.5 ± 0.1 µg equiv./ml and midnight value (0.3 ± 0.1 µg equiv./ml) at a P level of 0.05 and 0.005 respectively.
(● female; ■ male; —— mean; ----- SEM).

Table V.1.
AFBP, GH, cortisol and glucose were determined throughout a 24-hour period in a 10-year old girl (I) with idiopathic GH deficiency and in a 12.5-year old girl (II)with recent diabetes mellitus and treated with i.v. crystalline insulin.

Pat. I (♀; age: 10 yrs.) Dx: GH deficiency

t hrs.	AFBP µg equiv./ml	GH l U/L	cortisol µmol/L	glucose mmol/L
08.00	2.0	1	0.5	-
11.00	0.5	3		
14.00	0.4	4		
16.00	0.3	2	0.15	-
20.00	0.5	2		
22.00	0.6	0.5	0.04	-
01.00	0.6	3		
03.00	1.7	3.5	0.23	-
05.00	1.5	5		
08.00	1.9	1	0.30	-

Pat. II (♀; age: 12.5 yrs.) Dx: diabetes mellitus type I. Rx: crystalline, insulin i.v.

08.45	2.0	4	0.36	16.4
09.45	1.6	2	0.2	15.9
11.30	0.6	15	0.08	13.8
14.00	0.7	5	0.16	13.5
16.30	0.7	12	0.12	13.3
19.30				10.0
23.00	1.4	3	0.04	13.2
03.00	1.3	4	0.04	-

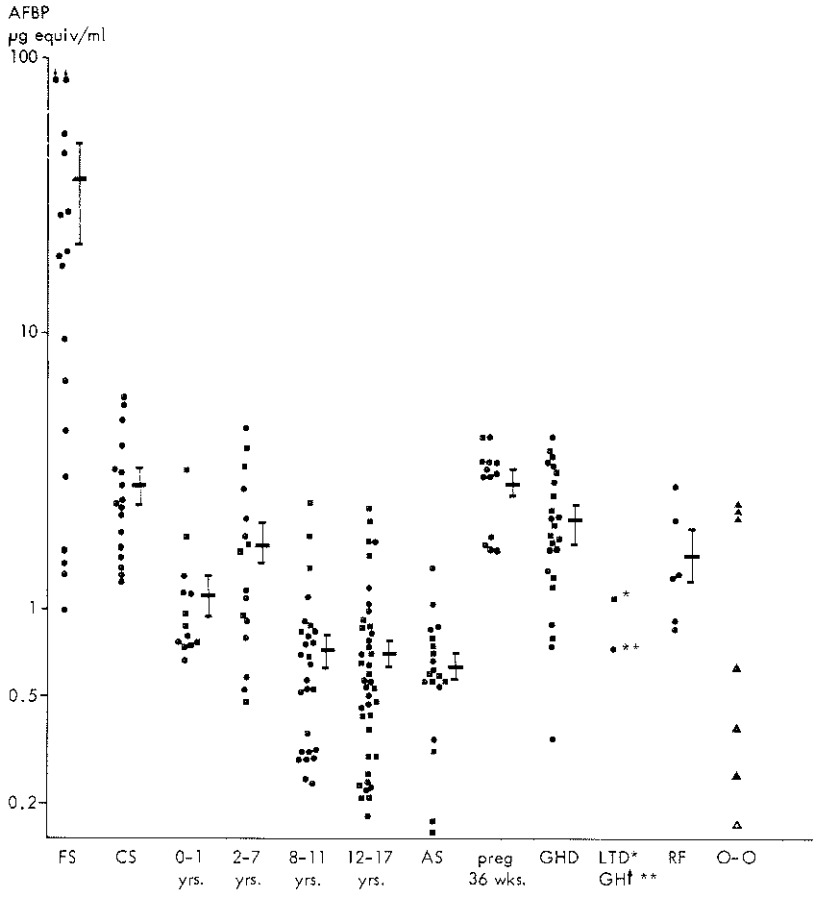


Fig. V.3.

AFBP concentrations plotted on a semi-logarithmic scale in native serum or plasma.

FS = fetal serum (18-22 weeks of gestation); CS = cord serum (32-40 weeks of gestation); children of 0-1, 2-7, 8-11, 12-17 years with non-endocrine illnesses.

AS = adult serum; preg = healthy pregnant women at 36 weeks of gestation; GHD = GH deficiency; RF = renal failure; LTD = Laron type dwarfism; GH↑ = GH excess; O-O = Orang-Outang (▲mother; ▲child); Δ=other animals (see text). ● = female; ■ = male. ⊥ = mean ± SEM.

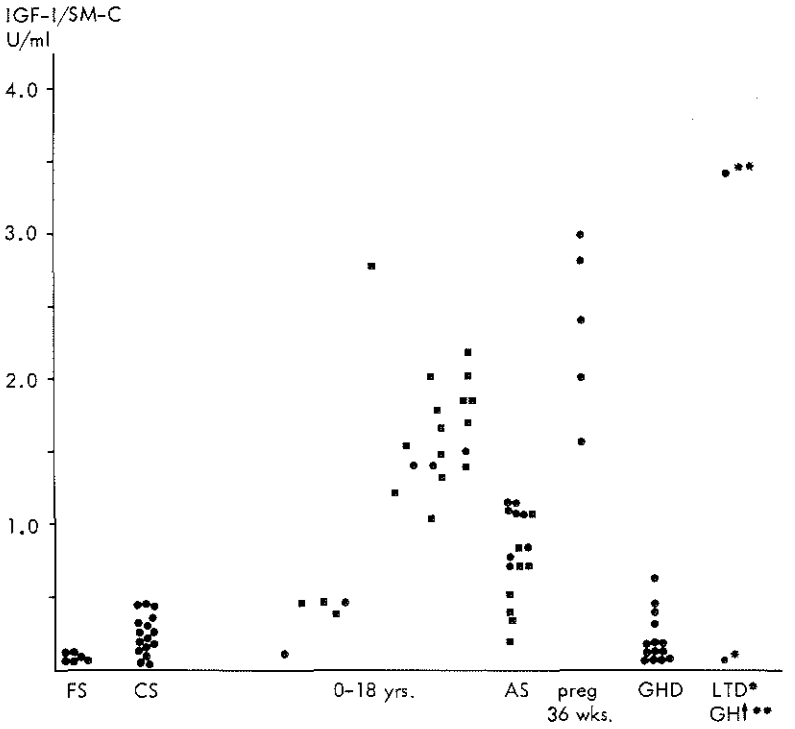


Fig. V.4.
IGF-I/SM-C concentrations in native plasma (determined by H.J. Guyda, McGill University, Montreal).
The values of healthy children of 0-18 years were obtained by H.J. Guyda, McGill University, Montreal (unpublished observations). For an explanation of abbreviations see legend figure V.3.

V.3.2. Conditions studied

V.3.2.a. Diurnal variation

Plasma AFBP values in 8 adults were highest early in the morning, declining during the day (fig. V.2.). A patient with recent diabetes mellitus and with intact GH and cortisol secretion, as well as a girl with GH deficiency and with a normal cortisol diurnal rhythm showed the same pattern (table V.1.).

V.3.2.b. Age dependence

The mean (± SEM) AFBP value in fetal serum was 36.7 ± 15.7 µg equiv./ml (n = 17). In cord serum this value was 2.8 ± 0.4 µg equiv./ml (n = 19). The difference is statistically not significant. In postnatal serum, the AFBP value gradually fell to adult levels of 0.6 ± 0.07 µg equiv./ml (n = 19). There was no difference between samples from males and females. IGF-I/SM-C values, determined in the same samples, were hardly detectable in fetal serum (mean 0.07 + 0.01 U/ml (n = 6)). In cord serum the mean value was 0.22 ± 0.04 U/ml (n = 12). In the age range of 0-18 years, there was a gradual rise of IGF-I/SM-C values. In adults SM-C values were generally lower than in pubertal individuals (fig. V.3., 4.).

V.3.2.c. GH dependence

The mean AFBP value in serum from children with GH deficiency (mean age 11.7 years, range 6.5-17 years; n = 23) was 2.0 ± 0.2 µg equiv./ml, significantly higher than

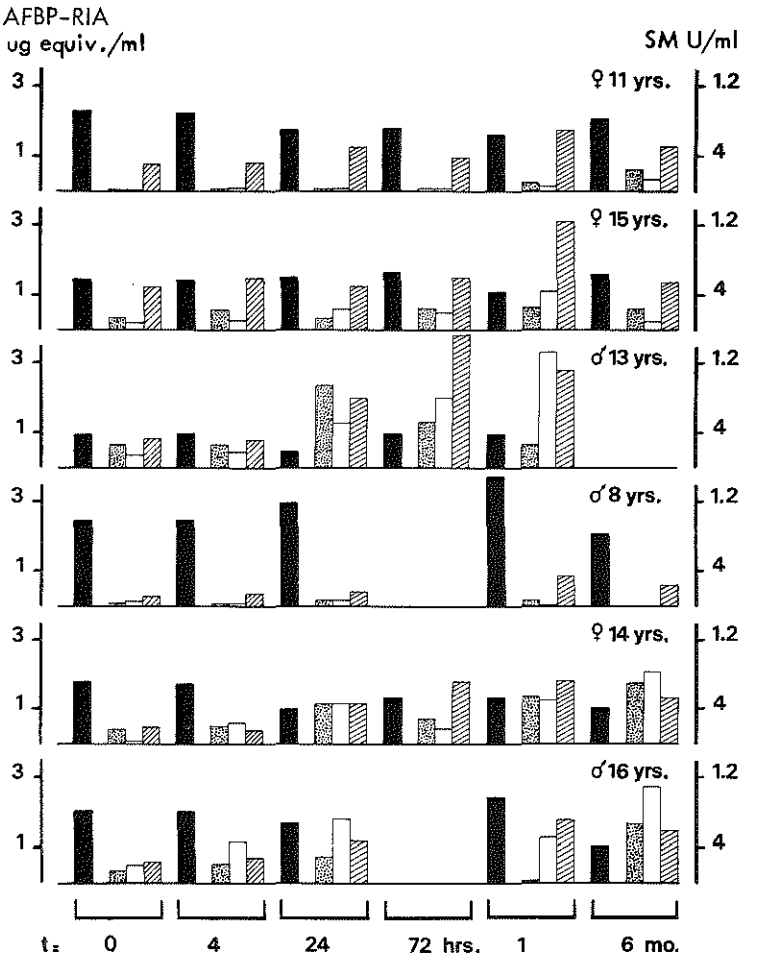


Fig. V.5.
The effect of GH treatment on SM and AFBP serum values in 6 patients at 4, 24 and 72 hours after a single injection of GH and after 1 and 6 months of continuous GH treatment.
AFBP values are indicated on the ordinate, left panel. SM values are indicated on the ordinate, right panel:
▒ = total immunoreactive SM, determined by RIA (Bala and Bhaumick, 1979).
□ = IGF-I/SM-C, determined by RIA (Guyda et al., 1981).
▨ = IGF-II, determined by RRA (Posner et al., 1977).
■ = AFBP, determined by RIA.

that found in the control groups in the age range of 7-11 years ($P \leq 0.05$) and 11-17 years ($P \leq 0.05$). As expected, SM values were markedly decreased (mean 0.21 $\pm$ 0.06 U/ml).

In 3 patients with GHD and on GH treatment for 9-24 months, the effect of a single i.m. injection of GH is given in table V.2.. In patient IV, a substantial rise of SM was found 20 hours after the GH injection. AFBP values showed a decrease. In the other two patients, no substantial rise of SM occurred following a single GH injection. AFBP morning values were increased. In 6 children with GH deficiency, AFBP and SM values were measured before and 4, 24 and 72 nours after one injection of GH (5 U) and then after 1 and 6 months of chronic GH treatment (dosage: 0.1

Table V.2.
The effect of a single i.m. injection of GH (5 mg, Nanormon ®) in 3 patients with GH deficiency on chronic GH treatment for 9-24 months

day	time hrs.	AFBP µg equiv./ml	IGF-I/SM-C ortho U/ml	GH 1U/L	Rx: GH (Nanormon ®) 5 mg i.m.
Pat. III	(♂; age: 15 yrs.; Tanner P_1G_1)				
1	17.00	0.40	0.11	1	
	17.30				x
2	08.30	1.9	0.17	0.5	
	16.00	0.8	0.15	0.5	
Pat. IV	(♂; age: 14 yrs.; Tanner P_1G_1)				
1	17.00	0.6	0.3	2	
	20.00				x
2	08.00	0.5	0.63	32	
	12.00	0.5	0.62	3	
	14.00	-	-	0.5	
	16.00	0.4	1.0	1	
	20.00	0.2	0.57	0.5	
Pat. V	(♀; age: 1,5 yrs.)				
1	18.00	0.7	0.23	0.5	
	18.30				x
2	09.00	3.0	0.14	1	

U/kg - 3x/week) (data taken from Dean et al., 1982). The SM response to GH varied considerably, and in these 6 patients no correlation between AFBP and SM values was found. In the adult with Laron dwarfism, the SM-C value was very low whereas the AFBP value was in the upper adult range. In the 14-year old girl with GH excess, the SM-C value was markedly elevated and AFBP in the normal range for age.

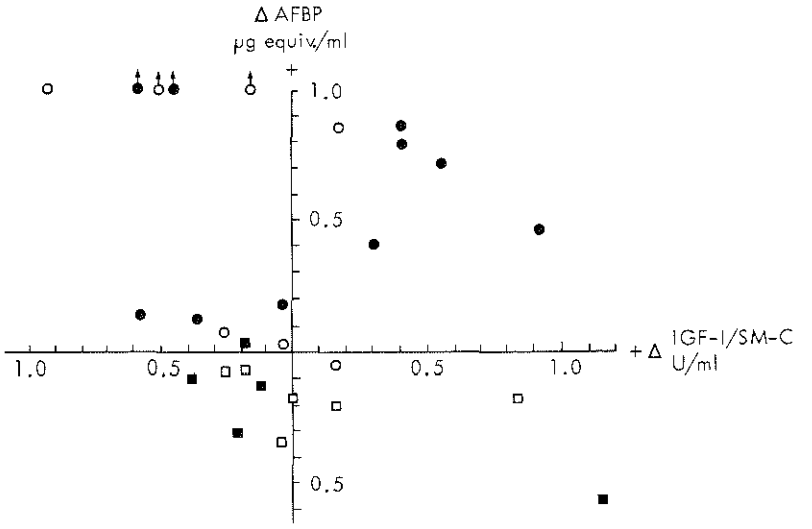


Fig. V.6.
The effect of sex-steroid treatment on SM and AFBP values in adolescents with tall stature.
On the abscissa the change of SM serum values during sex-steroid treatment is plotted. The ΔSM is defined as the SM plasma concentration at 2-4 months (● = ♀; ■ = ♂) or 5-8 months (○ = ♀ ; □ = ♂) minus the value obtained at the beginning of treatment. Similarly, on the ordinate the change of AFBP values in the same serum samples is indicated.

V.3.2.d. Sex-steroid dependence

In the adolescents with tall stature, treated with supraphysiological doses of sex-steroids, the change of SM values was highly variable (fig. V.6.). In the girls on estrogen treatment, AFBP values showed an increase whereas in the boys, treated with androgens, a slight decrease was noted.

V.3.2.e. Other physiological and pathological conditions

In 6 children with end stage renal failure and on hemodialysis treatment, the mean serum AFBP value was 1.6 $\pm$ 0.3 µg equiv./ml (fig. V.3.). Of 3 patients, 1 ml of serum was chromatographed on a 1 x 100 cm Sephadex G-200 column at pH 7.4, as described in chapter IV. AFBP-RIA activity was measured in the individual fractions. Peak activity was found only at K_{av} 0.55-0.75, and the calculated total AFBP content agreed with whole serum values (data not shown). In serum of pregnant women (36 weeks of gestation), AFBP and IGF-I/SM-C values were elevated: AFBP: mean 2.8 $\pm$ 0.3 U/l (n = 12), IGF-I/SM-C: mean 2.2 $\pm$ 0.3 U/l (n = 4) (fig. V.4.).

V.3.2.f. Other species

In undiluted serum of rats, rabbits and one elephant as well as in bovine and porcine serum, AFBP-RIA activity was at or below the limit of detection (fig. V.3.). However, in serum of 3 orang-outang mothers and their 3 children (age: 7-18 months), AFBP values were readily detectable.

V.3.2.g. In vitro studies

No active AFBP production could be demonstrated in cell cultures of preterm and term placentae. In supernatant of a hepatoma cell line in culture, immunoreactive AFBP was readily detectable: 1.3 µg equiv./ml. The SM-RIA activity was below the limit of detection.

V.4. Discussion

As AFBP was detectable in fractions of pre- and postnatal serum following Sephadex G-200 chromatography (chapter IV), a simple and yet reliable method for quantitation of serum AFBP was desirable. The dilution curve of native serum, at dilutions of 1:20 and up, ran parallel to the dilution curve of standard series to which 100 µl of a 1% solution of bovine serum had been added. In interpreting the results of AFBP determinations in native serum at low dilutions, one has to take into account the sensitivity limitations of the radioimmunoassay as a result of a final dilution of the antiserum (1:5000) and the low concentration of AFBP in terms of protein in postnatal serum (see chapter IV). Still, native serum content or plasma values concurred with the sum of the individual fractions, following Sephadex G-200 chromatography (data not shown).

The AFBP serum concentration appears to be influenced by several factors. First of all, AFBP values vary during the day, as illustrated in fig. V.2., suggesting the presence of a circadian rhythm sensitive regulatory mechanism, operative for pituitary hormones. A practical consequence is that comparison of serum concentrations would serve no useful purpose unless the time of sampling is known. IGF-I/SM-C values were not measured in these samples, but a de-

cline of serum SM-C concentrations after the onset of sleep has been reported (Minuto et al., 1981).

Secondly, with the increase of age, AFBP values in pre- and postnatal serum show a gradual decline (fig. V.3.). The opposite holds true for IGF-I/SM-C values (fig. V.4.), as has been reported by various authors (a.o. Furlanetto, 1980; Rosenfeld et al., 1981; Guyda et al., 1981).

Thirdly, the results of sex-steroid treatment of adolescents with tall stature suggest that AFBP values increase during estrogen treatment and decline during androgen treatment, a phenomenon well-known for several plasma proteins, such as sex-hormone binding globulin and ceruloplasmin (Bergink et al., 1976; Drop et al., 1981). The IGF-I/SM-C response to sex-steroid treatment is highly variable (fig. V.6.).

Finally, AFBP values are significantly higher in patients with GH deficiency than in age-matched controls. Preliminary studies indicate that during GH treatment AFBP concentrations decline whenever SM values rise (fig. V.5., table V.2.). Hintz described a similar paradoxical correlation between a nonsaturated, acid-stable SM binding protein with an apparent molecular weight of 40,000 and SM-C values before and after GH treatment of GH-deficient patients (Hintz et al., 1981). This SM binding protein has also been demonstrated in cord serum (Borsi et al., 1982).

By stimulating the production of SM itself and possibly of carrier proteins as well, GH is undoubtedly the single most important hormonal regulator of the SM concentration in serum. In addition, age, sexual differences, pubertal stage and particularly the nutritional status are all known to influence the SM-C concentration. The exact mechanism, by which these factors exert their influence on SM-C serum concentrations, is at present unknown and may well differ from the one applicable to AFBP. Therefore, it should

not be surprising that no statistically significant correlation was found between AFBP and IGF-I/SM-C values, measured in the same samples of pre- and postnatal serum.

In serum of pregnant women (36 weeks of gestation), both IGF-I/SM-C and AFBP values were elevated as compared to those in adult controls. This, again, points to factors other than GH, capable of influencing SM and AFBP serum values (Merimee et al., 1982[b]). In serum of uremic patients on hemodialysis, SM values were not measured, but decreased IGF-I/SM-C and increased IGF-II values have been reported by several authors (a.o. Schiffrin et al., 1978; Goldberg et al., 1982). Goldberg found increased concentrations of a 50,000 MW SM binding serum component. This fully agrees with our results, indicating AFBP values to be higher than those of age-matched controls, measured both in native serum and by Sephadex G-200 chromatography.

No active AFBP production could be demonstrated in cell cultures from preterm and term placentae. The liver is most likely the main site of production of SM binding proteins (Binoux et al., 1982). In supernatant of a hepatoma cell line in culture, immunoreactive AFBP was readily detectable. Moses reported on a human hepatoma cell line, HEP G2, secreting large quantities of a specific IGF carrier protein into serum-free medium. Similar to AFBP, this acid-stable protein has a molecular size of 30,000-50,000. It contains no glycoprotein moiety (Moses et al., 1983). The immunoreactivity of AFBP appears to be species-specific since, of the animal sera tested, only serum of the orang-outang reacted (fig. V.3.). We conclude that AFBP, a heat- and acid-stable SM binding protein, originally isolated from preterm amniotic fluid, shows a striking likeness to an unsaturated SMBP in human serum, as described by Hintz (1981).

CHAPTER VI

SUMMARY AND CONCLUSIONS

This thesis study was undertaken in order to investigate the nature and biological behavior of a somatomedin binding protein, identified in preterm amniotic fluid (AF).

Somatomedin (SM) is the generic designation applied to a family of serum peptide growth factors which are growth hormone dependent, stimulate incorporation of sulphate into cartilage, have insulin-like actions on nonskeletal tissues and increase mitosis in a wide variety of cultured cells. Thusfar two peptides have been fully characterized: insulin-like growth factor I (IGF-I), shown to be identical to somatomedin-C, and insulin-like growth factor II (IGF-II). IGF-I and IGF-II have a 62% aminoacid sequence identity and are 38-48% homologous with the A and B domains of human pro-insulin.

As outlined in chapter I, there are various indications that SM is involved in the humoral regulation of fetal growth. However, many questions as to its identity in fetal serum, its origin and mode of action remain unresolved. Growth hormone, prolactin, placental lactogen, insulin, glucagon and the nutritional status may all influence the regulation of SM during the fetal period.

SM circulates in plasma, bound to macromolecular weight carrier or binding proteins. In postnatal plasma, at least two forms have been recognized (150-200,000 and 40-50,000 MW). The precise function of these binding proteins is unclear. In addition to being carrier proteins, they may play an important role in the regulation of the biological effects of circulating SM.

In chapter II, the partial purification and characterization of an acid-stable protein with an isoelectric point of 4.7 and a presumed molecular weight of 35-40,000 are described. Furthermore, this protein, termed amniotic fluid binding protein (AFBP), is shown to specifically bind labeled SM (IGF-II) and inhibit the insulin- and somatomedin-like effects of IGF-II in various in vitro bioassays.

The purification procedure proved to be cumbersome and time-consuming. The yield was insufficient in terms of recovery and purity to allow a successful immunization of rabbits and further characterization. Therefore, an improved method for the isolation of AFBP from preterm AF was developed, with high speed gel filtration chromatography (HSGFC) and disc-gel electrophoresis as the final steps (chapter III). Details about the methodology of HSGFC are presented in appendix 1, chapter III. Purified AFBP migrated in analytical polyacrylamide gel electrophoresis systems as a single band with an apparent molecular weight of 35-40,000. AFBP probably is a glycoprotein, since it is recovered from a Concanavalin A column. Therefore, the molecular weight determination may be subject to error. The multiple bands, found during analytical polyacrylamide ampholine isoelectric focusing at pH 4.7-4.9, may represent heterogeneity in the degree of glycosylation of the AFBP molecule. As outlined in appendix 2, chapter III, the SM binding activity is heat-stable, irrespective of the hydrogen ion concentration. Thusfar, the final yield of purified AFBP was insufficient to allow either aminoacid analysis or sedimentation and equilibrium analysis by analytical ultracentrifugation.

There are various reasons for the limited yield. The AFBP content in preterm AF, in terms of microgram protein, is probably very low. The high SM binding activity found in preterm AF results from the high binding capacity of AFBP, as

preliminary competitive binding studies with labeled IGF-I and IGF-II indicate (kindly performed by H.J. Guyda, Montreal, and A.J. D'Ercole, Chapel Hill). The substantial losses can be accounted for by shoulder fractions and trailing. Although SM binding activity and immunoreactivity (see below) of partially purified AFBP are maintained following acid treatment and heating procedures at various pH levels, molecular instability in protein (HSA) depleted conditions is not ruled out. Also, nonspecific attachment of AFBP to tubing and glassware during the last purification steps is likely to be a cause of protein loss.

However, sufficient purified AFBP was obtained to immunize two rabbits. An antibody was raised in one rabbit (chapter IV). The antiserum was cleared of anti-albumin antibodies by affinity-chromatography. Monospecificity was suggested by the lack of cross-reactivity of a limited number of proteins, among which HSA, α-fetoprotein and some glycoproteins. Furthermore, immunoelectrophoresis of concentrated preterm AF and fetal serum in the presence of the antibody resulted in one precipitin line (silver staining).

The generation of a highly specific antibody directed against AFBP was the main initial objective of this study. Purification, quantitation as well as the study of ontogeny, of homology with known SM binding proteins and of biological behavior would all be greatly facilitated by the availability of such an antibody. At present, the antibody is used for final purification of AFBP by affinity-chromatography (study in progress).

A double antibody radioimmunoassay was developed. Purified AFBP was iodinated by the chloramine-T method, and dilutions of partially purified AFBP were designated as the standard, with the results expressed in μg equivalent protein/ml. The sensitivity of the assay was improved by adop-

tion of the nonequilibrium procedure. AFBP values were twice as high in preterm AF (gestational age of 16-22 weeks) as in term AF (38-42 weeks). As SM binding activity in AF, determined at different gestational ages, correlated with fetal renal maturation, AFBP was suggested to be present in serum (Chochinov et al., 1976; Drop et al., 1979). This was confirmed by measuring fractions of Sephadex G-200 chromatographed pre- and postnatal serum by AFBP-RIA. Activity eluted only at a volume corresponding to a molecular size of 35-40,000, identical to the elution volume of labeled AFBP. In addition, AFBP was found in urine of preterm infants. It is of specific interest that no AFBP-RIA activity was discovered in the large molecular weight range in serum, chromatographed either at neutral pH (7.4) or under protein dissociating conditions (pH 1-2). Fraction values generally were higher when serum had been acidified. Thus the AFBP-antibody dit not recognize the 150-200,000 MW SM binding proteins. The possibility, however, that AFBP and the 150-200,000 MW BP are related, has not been ruled out. When labeled SM and serum SM binding proteins were subjected to cross-linking procedures, followed by polyacrylamide gel electrophoresis, several bands, including one of 38,000 MW, were found. The AFBP-antibody will be used to determine AFBP antigenicity of the bands (study in progress in collaboration with A.J. D'Ercole, Chapel Hill). By measuring SM and SM binding activity in the individual fractions, we could confirm the observation by D'Ercole that at neutral pH the elution patterns of IGF-I/SM-C in fetal and cord serum differ (D'Ercole et al., 1980). In fetal serum of infants of less than 27 weeks of gestation, IGF-I/SM-C elutes at an apparent molecular weight of 40,000, whereas in cord serum the predominant elution occurs at a molecular weight of 150-200,000. Thus, with gestation progressing, the predominant form in which SM circulates in plasma, changes from an apparent molecular weight of 40,000 to 150-200,000, the

same found in normal adult sera. The 150-200,000 MW SM binding protein is believed to be under GH control, since SM binding and SM activity of 150-200,000 MW are diminished in serum of GH-deficient patients and restored when GH is replaced (Zapf et al., 1978; Copeland et al., 1980; White et al., 1981; Schalch et al., 1982). GH is present in significant concentrations in human fetal serum at 7-10 weeks of gestation (Gluckman et al., 1980). The absence of the 150,000 MW SM binding protein in mid gestation fetal serum might reflect fetal GH resistance or, more likely, immaturity of the mechanisms involved in the synthesis of SM binding proteins (D'Ercole et al., 1980).

Sara measured SM activity in human serum by radioreceptorassay, utilizing human fetal brain plasma membrane as matrix (Sara et al., 1981). Levels in fetal serum were found to be increased, not only compared with adult values, but also with SM values measured by RIA. On the basis of this discrepancy between SM values measured in fetal serum by RRA and RIA, it was postulated that in the human fetus an embryonic form of SM exists. At the end of the first half of gestation, this embryonic SM is present at high concentrations. During the second half of gestation, the values gradually fall as adult forms of SM begin being produced. However, in this study whole serum was tested and fetal serum was not subjected to another RRA. In chapter IV, the demonstration of substantial quantities of SM binding activity in fetal serum is presented. Therefore, the discrepancy between RRA and RIA SM values in fetal serum can be fully explained by interference in the RRA of SM binding proteins, such as AFBP, leading to spuriously high values.

Several recent observations in the rat have suggested a possible role for IGF-II in fetal growth. IGF-II levels are 20-100fold higher in fetal rat serum than in maternal

serum and decline within days after birth (Moses et al., 1980). IGF-I exhibits the reciprocal developmental pattern: low values in the early neonate, rising to adult levels by approximately 4 weeks of age (Daughaday et al., 1982[b]). This thesis study confirms that IGF-I values in human fetal serum are low. IGF-II values in fetal serum determined by RIA have not been reported on. However, in fetal or cord serum, decreased SM activity has been found, determined by various bioassays and radioreceptorassays in which IGF-II is reactive (Goodyer, 1981). Thus the question of the identity of fetal SM remains unresolved. The presence of large quantities of a specific SM binding protein (AFBP) in fetal serum is an additional indication that SM plays a physiological role during fetal growth.

As AFBP was detectable as one peak in fractions of pre- and postnatal serum following Sephadex G-200 chromatography, the AFBP-RIA was adapted in order to measure AFBP directly in native serum or plasma (chapter V). The dilution curve of native serum, at dilutions of 1:20 and up, ran parallel to that of the standard series to which 100 µl of a 1% solution of bovine serum had been added. Quite interesting was the observation that acid treatment of serum results in an improvement of parallelism at low dilutions. Furthermore, AFBP values generally were higher in acid-treated serum than in the same untreated sample. Acid treatment of Cohn fraction IV or serum (human, rat) shifted recovered SM binding proteins from 150,000 to approximately 40-60,000 MW forms (Hintz and Liu, 1980; Moses et al., 1979). Furlanetto (1980) reported on an acid-stable, 40,000 dalton subunit of the 150,000 MW SM binding protein. It is rather tempting to speculate that the AFBP-antibody recognizes these 40-60,000 MW subunits.

The AFBP serum concentration appears to be influenced by several factors. First of all, AFBP values vary during the day, suggesting the presence of a circadian rhythm sensitive regulatory mechanism, operative for pituitary hormones. A practical consequence is that comparison of serum concentrations is only useful if the time of sampling is known. SM values vary little throughout the day, although a decline of serum SM-C concentrations after the onset of sleep has been reported (Minuto et al., 1981).
Secondly, with the increase of age, AFBP values in pre- and postnatal serum show a gradual decline. The opposite holds true for SM values. This phenomenon remains unexplained. It has been estimated that less than 1% of SM circulates free of its carrier protein complex in serum (Zapf et al., 1980). The vast majority is bound to its binding proteins. In general, therefore, total SM is measured, following acid treatment or an extraction procedure in order to strip SM from its binding proteins. Similar to the change of serum thyroxine and cortisol concentrations reflecting thyroxine-binding globulin and cortisol-binding globulin content, a parallel shift of SM and AFBP concentrations would be expected. The unknown factor remains the quantitation of the 150-200,000 MW binding proteins.
Thirdly, the results of sex-steroid treatment of adolescents with tall stature suggest that AFBP values increase during estrogen treatment and decline during androgen treatment, a phenomenon well-known for several plasma proteins, such as sex-hormone binding globulin and ceruloplasmin.
Finally, an important observation was that AFBP values were significantly higher in patients with GH deficiency than in age-matched controls. As expected, GH treatment resulted in a rise of SM values. However, preliminary data indicate that AFBP values decline. Hintz described a simi-

lar paradoxical correlation between a nonsaturated, acid-stable SM binding protein with an apparent molecular weight of 40,000 and SM-C values before and after GH treatment of GH-deficient patients (Hintz et al., 1981). It is of specific interest to note that the large molecular weight SMBP is GH-dependent, in that low 150-200,000 MW binding activity in serum of GH-deficient patients increases to normal during GH treatment (Zapf et al., 1978; Copeland et al., 1980; White et al., 1981; Schalch et al., 1982). The correlation between GH, SM and its binding protein values in serum of patients with GH deficiency and other growth-related disorders will be the subject of further intensive study.

The liver is most likely the main site of production of SM binding proteins (Binoux et al., 1982). No active AFBP production could be demonstrated in cell cultures of preterm and term placentae. In supernatant of a hepatoma cell line in culture, immunoreactive AFBP was readily detectable. Moses reported on a human hepatoma cell line (HEP G2) secreting large quantities of a specific SM carrier protein (30-50,000 MW) into serum-free medium (Moses et al., 1983). Of particular interest will be in vitro studies of the mechanisms through which various hormones (or patient sera) influence the synthesis and release of AFBP in the human hepatoma cell line system.

Whereas somatomedin-like peptides, isolated from human and various animal sources, cross-react in their respective detection systems, notably radioreceptorassays and radioimmunoassays, the immunoreactivity of AFBP appears to be species-specific, since of the animal sera tested only serum of the orang-outang reacted.

Thusfar, the physiological role of the SM binding proteins is undefined. They prolong the serum half life of SM, which explains the relatively high and constant levels of SM in plasma as compared to those of other peptide hormones.

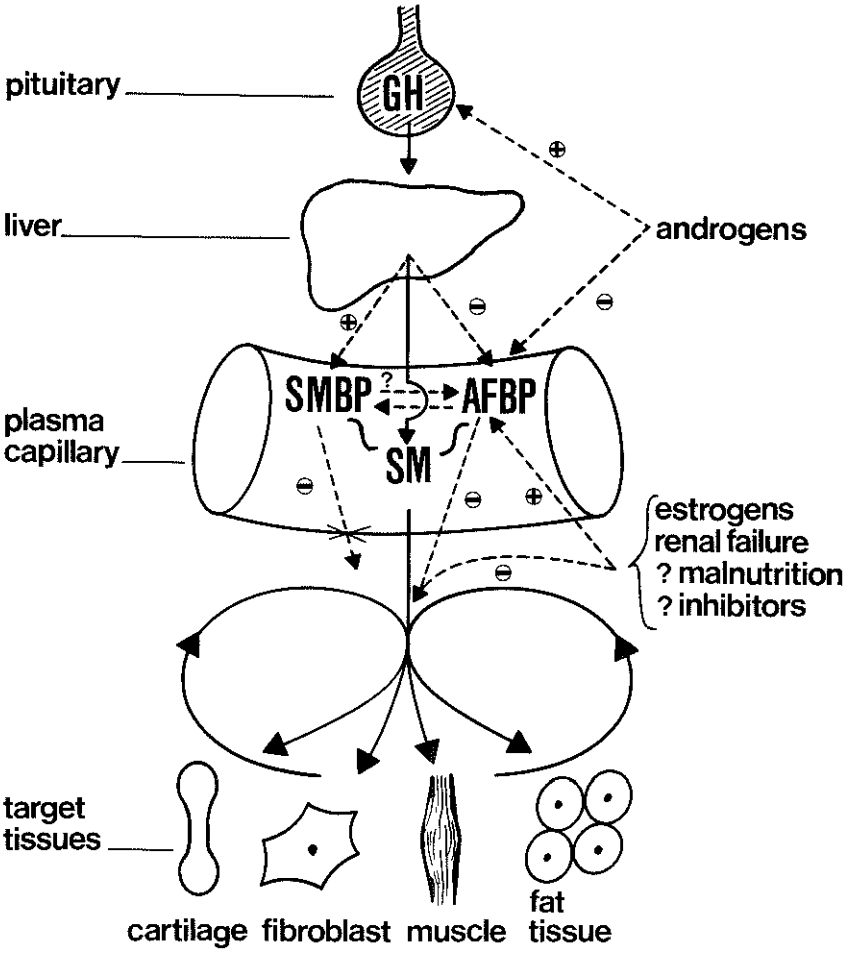


Fig. VI.1.
Schematic diagram of some hypothetical mechanisms involved in the regulation of SM, AFBP and the large MW SM binding proteins (SMBP), based upon data presented in chapter I, II, V (cf. fig. I.1.).

SM bound to binding proteins does not readily diffuse through the capillary walls (Meuli et al., 1978). Therefore, the SM binding protein complex cannot compete for the insulin receptor site, protecting the tissues from the potentially large, insulin-like effect of SM (Zapf et al., 1979). This specifically holds true for the large molecular weight binding proteins (150-200,000 MW). It is not certain whether this applies to AFBP as well, for it has been demonstrated that particles with a molecular weight of up to 40,000 can leave the vascular system (Garlick et al., 1970). Various factors are likely to influence the binding characteristics of the SM-AFBP-complex, such as pH and serum proteases (Chatelain et al., 1983), and the binding kinetics of SM at a cellular level (Rechler et al., 1980; Sara et al., 1983). Further study of these factors is called for in order to answer the question whether AFBP functions solely as an SM inhibitor by limiting SM bioavailability. Alternatively, AFBP may provide the growing organ with high and constant circulating levels of SM, necessary for the diffusion of SM into crucial avascular tissues, such as cartilage (Hintz and Liu, 1981).

A schematic diagram of some hypothetical mechanisms, involved in the regulation of SM, AFBP and the large MW SM binding proteins, is presented in fig. VI.1..

The role of SM in pre- and postnatal growth will not be fully understood unless the function of the SM binding proteins has been clarified. The development of the AFBP-radioimmunoassay may contribute to a further elucidation of the physiological importance of SM and the SM binding proteins in pre- and postnatal growth.

SAMENVATTING EN CONCLUSIES

In dit proefschrift wordt de methode beschreven waarmee een somatomedine bindend eiwit uit preterm vruchtwater gezuiverd en gekarakteriseerd werd. Derhalve werd dit eiwit "Amniotic Fluid Binding Protein" (AFBP) genoemd. Nadat tegen dit eiwit een konijneantiserum bereid was, werd een specifieke bepalingstechniek voor AFBP volgens de radioimmunoassay methode ontwikkeld. Daardoor werd het mogelijk het biologisch gedrag van dit eiwit te bestuderen.

Met de term somatomedine (SM) wordt een groep serumeiwitten aangeduid die de groei bevorderen en de volgende eigenschappen bezitten: zij zijn van het groeihormoon afhankelijk en stimuleren de opname van sulfaat in het kraakbeen. Zij vertonen bovendien insuline-achtige kenmerken en versnellen de celdeling in vitro en in vivo. Tot dusver zijn twee eiwitten volledig gekarakteriseerd: "Insulin-like growth factor" (IGF) I en II. Daarnaast komen in het serum van mens en dier waarschijnlijk nog andere, onvolledig omschreven vormen voor.

In hoofdstuk I wordt aangetoond dat er goede aanwijzingen zijn dat SM een belangrijke rol bij de hormonale regulatie van foetale groei speelt. De aard en het werkingsmechanisme van foetaal SM zijn evenwel onopgehelderd. Het is zeer aannemelijk dat verschillende hormonen, alsmede voedingsfactoren de werking van SM gedurende de foetale periode reguleren.

In plasma komt SM vrijwel niet in ongebonden vorm voor, echter wel gebonden aan transport- of bindende eiwitten met een molecuulgewicht van ongeveer 150-200.000 en 40-50.000. De functie van deze eiwitten is nog onduidelijk.

Het is denkbaar dat zij een belangrijke rol spelen bij de regulatie van de biologische werkzaamheid van SM.

In hoofdstuk II wordt de gedeeltelijke zuivering van AFBP uit preterm vruchtwater beschreven. Dit eiwit bindt specifiek SM, zoals IGF-II, en remt de biologische werkzaamheid van SM in vitro. Het iso-electrisch punt van dit zuurbestendige eiwit bedraagt 4.7 en het molecuulgewicht is geschat op 35-40.000.

In hoofdstuk III wordt uiteengezet hoe een bepalingsmethode (de "charcoal binding assay" (CBA)) ontwikkeld werd waarmee op een snelle en eenvoudige, zij het weinig gevoelige wijze de bindende werkzaamheid van SM aangetoond kon worden. Met behulp van onder andere hogedruk-vloeistofchromatografie en gelelectroforese werd een zekere hoeveelheid gezuiverd AFBP verkregen met het doel om deze nader te karakteriseren en er konijnen mee te immuniseren. De methode van de hogedruk-vloeistof-chromatografie wordt uitvoerig in appendix 1, hoofdstuk III beschreven. De opbrengst van gezuiverd AFBP bleek onvoldoende te zijn voor een analytische ultracentrifugatie studie en een aminozuuranalyse. Wel kon worden aangetoond dat AFBP een glycoproteine en bij een van 2 tot 8.5 variërende pH hittebestendig is (appendix 2, hoofdstuk III).

In hoofdstuk IV komt de bereiding van het AFBP antiserum aan de orde. Storende anti-albumine antilichamen werden d.m.v. een affiniteitschromatografie verwijderd. Dat het antilichaam specifiek tegen AFBP gericht is, bleek onder meer uit immunoelectroforese proeven. Met behulp van het AFBP antiserum werd een bepalingstechniek volgens de radioimmunoassay methode (RIA) uitgewerkt. Van een aantal serumeiwitten, zoals albumine, α-fetoproteine en enkele glycoproteinen, werd aangetoond dat zij geen kruisreactie

vertonen.
AFBP waarden bleken tweemaal zo hoog in preterm vruchtwater (zwangerschapsduur 16-22 weken) als in à terme vruchtwater (zwangerschapsduur 38-42 weken).
Ook in op een Sephadex G-200 kolom gescheiden foetaal en postnataal serum werd AFBP aangetoond, en wel in fracties die met een molecuulgewicht van 35-40.000 overeenkomen.
Er werden geen aanwijzingen gevonden dat het AFBP antilichaam tevens de bindende eiwitten met een groot molecuulgewicht herkent.

In fracties van op een kolom gescheiden foetaal en à terme navelstrengbloed werden SM en de bindende werkzaamheid van SM gemeten. Aldus kon de waarneming van D'Ercole (1980) bevestigd worden dat in vóór de 27ste week van de zwangerschap verkregen foetaal serum de bindende werkzaamheid van SM met een molecuulgewicht van $\pm$ 40.000 overeenkomt en in navelstrengbloed (à terme zwangerschap) voornamelijk de bindende werkzaamheid bij een molecuulgrootte van 150-200.000 gevonden wordt. Dit is opmerkelijk omdat de SM-bindende eiwitten met een molecuulgewicht van 150-200.000 van het groeihormoon (GH) afhankelijk zijn en reeds zeer vroeg in de zwangerschap (7^{e}-10^{e} week) hoge GH spiegels in het serum gevonden worden. Derhalve zou er sprake kunnen zijn van ongevoeligheid voor GH. Waarschijnlijker echter is, dat pas in de loop van de zwangerschap het mechanisme dat de synthese reguleert tot ontwikkeling komt.

Sara (1981) heeft de werkzaamheid van SM in onbehandeld foetaal serum van de mens bepaald waarbij zij van een radioreceptorassay (RRA), met celmembranen van foetale hersencellen als receptor, gebruik maakte. Zij constateerde een grote discrepantie tussen de met behulp van deze radioreceptorassay en de met een voor SM specifieke radioimmunoassay bepaalde waarde van SM in foetaal serum. Der-

halve postuleerde zij dat er een embryonale vorm van SM bestaat die niet met de gangbare RIA methoden , maar wel in de bovengenoemde RRA gemeten kan worden.

Hoofdstuk IV toont aan dat AFBP in aanzienlijke hoeveelheden in foetaal serum voorkomt. Het is welbekend dat SM-bindende eiwitten de bepaling van SM d.m.v. de radioreceptorassay kunnen verstoren, aangezien zij met de receptor wedijveren om SM te binden. Daarom zou de discrepantie tussen de met de RRA en met de RIA gemeten spiegels van SM in onbehandeld foetaal serum het gevolg kunnen zijn van de aanwezigheid van relatief grote hoeveelheden AFBP.

Er zijn goede aanwijzingen dat IGF-II in de rat de rol van foetaal SM vervult. IGF-II waarden in foetaal ratteserum zijn 20-100 maal hoger dan in moederlijk serum en dalen binnen enkele dagen na de geboorte. IGF-I spiegels vertonen juist het omgekeerde patroon: laag bij de pasgeborene, geleidelijk stijgend tot volwassen waarden op de leeftijd van 4 weken. Ook in menselijk foetaal bloed zijn IGF-I waarden laag. Met bioassays en radioreceptorassays bepaalde IGF-II gehaltes in navelstrengbloed zijn evenwel ook laag.

Dus de vraag of een embryonaal SM bij de mens bestaat blijft vooralsnog onbeantwoord.

In hoofdstuk V worden de resultaten genoemd van een aantal waarnemingen die aan de hand van AFBP bepalingen in onbehandeld serum of plasma gedaan zijn. De verdunningscurve van onbehandeld serum, mits meer dan 1 : 20 verdund, bleek parallel te lopen aan de standaard verdunningsreeks, waaraan 1% runderserum toegevoegd was. Een voorbehandeling van het serum met HCl (0.6 M), gevolgd door neutralisatie met NaOH (0.6 M), verbeterde het parallellisme bij lage verdunning. AFBP bepalingen in met zuur behandeld serum waren in het algemeen hoger dan in onbehandeld serum. Het

is reeds bekend dat zuurbehandeling van Cohn fractie IV of serum tot gevolg heeft dat de bindende werkzaamheid van SM van een molecuulgewicht van 150-200.000 naar 40-60.000 verschuift. Het SM-bindend eiwit met een molecuulgewicht van 150-200.000 bevat een zuurbestendige component met een molecuulgewicht van ± 40.000. In samenwerking met dr. A.J. D'Ercole (Chapel Hill) zal met behulp van het antiserum de samenhang tussen de groot- en kleinmoleculaire SM-bindende eiwitten en AFBP in menselijk serum nader bestudeerd worden.

De concentratie van AFBP in serum wordt door verschillende factoren beïnvloed:

1. AFBP serum waarden zijn 's ochtends hoger dan 's avonds. Dit zou erop kunnen wijzen dat er een regulatiemechanisme bestaat dat aan een 24-uursritme onderhevig is zoals dat bijvoorbeeld voor een aantal hypofyse hormonen geldt. Daarentegen zijn SM serum spiegels per etmaal vrij constant alhoewel een geringe daling kort na het inslapen waargenomen is.
2. In pre- en postnataal serum nemen AFBP waarden met toenemende leeftijd af. Het omgekeerde doet zich bij SM spiegels voor. Een afdoende verklaring is nog niet voorhanden. Geschat wordt dat minder dan 1% van het in bloed aanwezige SM in ongebonden vorm circuleert. De overige 99% is gebonden aan de bindende eiwitten. Daarom wordt meestal totaal SM gemeten, hetgeen betekent dat het serum eerst met HCl behandeld of geëxtraheerd wordt zodat SM van de bindende eiwitten gescheiden kan worden. Zoals thyroxine en cortisol concentraties de gehaltes van thyroxine-bindend globuline en cortisol-bindend globuline weerspiegelen, zo zou men ook een parallel beloop van SM en AFBP concentraties kunnen verwachten. De onbekende factor blijven echter de bindende eiwitten met een groot molecuulge-

wicht (150-200.000) die tot dusver niet te quantificeren zijn.

3. De resultaten van sexsteroïd behandeling van adolescenten met een grote lengte suggereren dat AFBP waarden onder invloed van oestrogenen toenemen en onder invloed van androgenen afnemen. Ditzelfde patroon vertonen ook verscheidene plasma eiwitten zoals bijvoorbeeld het sexhormoon-bindend eiwit en ceruloplasmine.
4. Tenslotte is de waarneming van betekenis dat AFBP waarden in serum van patiënten met GH-deficiëntie significant hoger dan die in serum van controlepatiënten van overeenkomstige leeftijd zijn. Er zijn voorlopige aanwijzingen gevonden dat AFBP serum waarden in deze patiënten tijdens GH behandeling dalen, terwijl zoals bekend SM spiegels stijgen. Hintz (1981) heeft deze zelfde paradoxale correlatie tussen een onverzadigd, zuurbestendig, SM-bindend eiwit met een molecuulgewicht van 35-40.000 en SM waarden in serum van GH-deficiënte patiënten tijdens GH behandeling beschreven . In dit verband is het van belang op te merken dat de SM-bindende eiwitten met een molecuulgewicht van 150-200.000 van het groeihormoon afhankelijk zijn. Immers, lage concentraties van deze eiwitten in serum van GH-deficiënte patiënten nemen toe tijdens GH behandeling. De correlatie tussen GH, SM en de SM-bindende eiwitten in serum van patiënten met GH-deficiëntie en andere groeistoornissen zal onderwerp zijn van verder intensief onderzoek.

De SM-bindende eiwitten worden waarschijnlijk in de lever aangemaakt. In het supernatant van een hepatomacellijn in kweek is AFBP aangetoond. Dit is in overeenstemming met de bevindingen van Moses (1983) dat een menselijke hepatomacellijn een SM-bindend eiwit met een molecuulgewicht van $\pm$ 40.000 produceert. Een interessante en belang-

Erratum: pag.149 regel 11
verlagen, lees verlengen

rijke vervolgstudie zou de bestudering van het mechanisme kunnen zijn waarmee verschillende hormonen of serum van patiënten de synthese van AFBP in de menselijke hepatoma-cellijn in vitro reguleren.

De immunoreactiviteit van AFBP is vermoedelijk species-gebonden. Met behulp van de radioimmunoassay is AFBP alleen aantoonbaar in serum van de orang-oetang en niet in serum van het konijn, de rat, het varken en het rund.

De fysiologische functie van de SM-bindende eiwitten is nog onduidelijk. Het staat vast dat de bindende eiwitten de serum halfwaardetijd van SM ~~verlagen,~~ verlengen en dit verklaart waarschijnlijk de relatief hoge en constante SM serum spiegels in vergelijk met die van andere peptide hormonen. Aan de bindende eiwitten gebonden SM diffundeert niet door de wand van de bloedcapillairen heen. Derhalve kan het SM-bindend eiwitcomplex de insulinereceptor niet in beslag nemen en beschermt het bindend eiwit de weefsels tegen de mogelijk aanzienlijke insuline-achtige werkzaamheid van SM. Dit geldt zeker voor de eiwitten met een groot molecuulgewicht (150-200.000). Maar aangezien deeltjes met een molecuulgewicht tot $\pm$ 40.000 het vaatbed mogelijk wel kunnen verlaten, bestaat er enige twijfel of dit ook voor AFBP opgaat.

Nader onderzoek is nodig naar het mechanisme waardoor SM aan de bindende eiwitten en aan de celmembraan van het eindorgaan gebonden wordt. Deze studie zou een antwoord kunnen geven op de vraag of AFBP uitsluitend als remmer van de biologische werkzaamheid van SM optreedt dan wel als een transporteiwit fungeert, dat het lichaam van constante spiegels van circulerend SM voorziet.

Met de ontwikkeling van een specifieke bepalingsmethode voor AFBP, zoals in dit proefschrift beschreven is, is ge-

poogd een bijdrage te leveren aan de opheldering van de fysiologische betekenis van SM en de SM-bindende eiwitten tijdens het pre- en postnatale groeiproces.

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ACKNOWLEDGEMENTS

To all who have assisted in the completion of this thesis study, I wish to convey my sincere feelings of gratitude.

I thank my parents who have expressed warm interest and provided support throughout my years of undergraduate training and medical school.

Thanks are due to dr. W.K. Schubert. He hired an unknown Dutch intern "over the phone" and offered an intensive and unique pediatric training and teaching program at the Children's Hospital Medical Center, Cincinnati, Ohio, USA.

I also owe thanks to dr. E. Colle who introduced me to the vast field of clinical pediatric endocrinology.

My genuine appreciation is directed to dr. H.J. Guyda. His guidance, encouragement and incessant "transcontinental" support over the years have been of immeasurable value to me.

I thank prof. dr. H.K.A. Visser for his support and for enabling me to combine clinical pediatric endocrinology with laboratory research.

To prof. dr. J.L. Van den Brande I am grateful for many pleasant sessions affording an opportunity for him and dr. S. Van Buul-Offers to offer valuable "feedback". His critical review of the manuscript is greatly appreciated.

I gratefully recognize prof. dr. H.G. van Eijk's valuable advice regarding the characterization studies and his comments and suggestions.

To Mrs. J. Parodo and Mrs. D.J. Kortleve I am very much indebted. Mrs. Parodo taught me basic laboratory techniques. Without the unabated dedication and enthusiasm of Mrs. Kortleve this thesis study would simply not have been written.

I gratefully acknowledge:
- dr. W. Hackeng, endocrine laboratory, Bergwegziekenhuis, Rotterdam, for performing all iodinations;
- prof. dr. M. Niermeyer, department of clinical genetics, Erasmus University, Rotterdam, and dr. J. Verjaal, department of clinical genetics, University of Amsterdam, for supplying gallons of uncontaminated preterm amniotic fluid, the very basis of this study;
- dr. R. Rozing and dr. W. Hesse for helping with the immunization of the rabbits;
- prof. dr. H.J. Degenhart for procuring statistical advice;
- Mrs. E.A.D.M. Nelemans-van den Broek for correcting the list of references;
- Mrs. M. de Bruijne-Scharrenberg, assisted by Mrs. A. de Reus, for typing the numerous manuscript versions;
- Mr. L. Baars and Mr. J. de Kuyper, assisted by Mrs. H. Versprille and Mrs. E. Hinderdoel, for drawing the figures and designing the layout.

Last but not least, I would like to thank my wife Henriette for correcting the entire text of this thesis study and for enduring the countless seclusive hours required for the completion of this study.

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