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**Hormonal Regulation and Immunological Characterization  
of Insulin-Like Growth Factor (IGF)-Binding Proteins  
in Cultured Rat hepatocytes**

**Yannopoulos Constantin**

**A Thesis**

**in**

**The Department**

**of**

**Chemistry and Biochemistry**

**Presented in Partial Fulfillment of the Requirements  
for the Degree of Master of Science at  
Concordia University  
Montréal, Québec, CANADA**

**SEPTEMBER 1991**

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## ABSTRACT

### **Hormonal Regulation and Immunological Characterization of Insulin-Like Growth Factor (IGF)-Binding Proteins in Cultured Rat hepatocytes**

**Yannopoulos Constantin**

The major organ for production of insulin-like growth factor (IGF)-I and IGF-binding proteins (IGF-BPs) is the liver. IGF-I is thought to be an important mediator of growth hormone (GH) action and is influenced by GH. From previous studies, both *in vivo* and *in vitro* GH effects on liver IGF-BPs were masked by the presence of endogenous GH in the circulation and in serum conditions for cellular growth respectively.

We have maintained primary cultured adult rat hepatocytes in serum-free conditions and studied the role of GH and glucagon in the regulation of IGF-I and IGF-BP secretion. GH inhibited the secretion of IGF-BPs whereas glucagon stimulated their production. This differential regulation was not seen for IGF-I where both hormones were stimulatory. The inhibitory action of GH on IGF-BP secretion was reversed by glucagon.

Western blotting (WB) permitted the identification of two bands which migrated between the region of  $M_r = 25,000$  and 30-34,000 daltons (Da). Immunological studies permitted the identification of two distinct IGF-BPs: (i) IGF-BP-1 and (ii) IGF-BP-2 of molecular weights of (31-32,000 Da) and (35-36,000 Da) respectively within the 30-34 kDa complex seen by WB. The small molecular weight BP ( $M_r = 25,000$ ) had no immunoreactivity to neither antibody suggesting a different species. Dose-dependent inhibition studies proved that binding proteins have unique specificity for IGFs.

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## TABLE OF CONTENTS

|   | PAGE |
|---|------|
| ABSTRACT                                      | III  |
| ACKNOWLEDGEMENTS                              | IV   |
| TABLE OF CONTENTS                             | V    |
| LIST OF FIGURES                               | VIII |
| LIST OF TABLES                                | XI   |
| 1. INTRODUCTION                               | 1    |
| 1.1 The Neuroendocrine Axis of Control        | 1    |
| 1.2 Regulation of Growth Hormone<br>Secretion | 3    |
| 1.3 Growth Hormone and IGFs                   | 4    |
| 1.4 Insulin-like Growth Factors (IGFs)        | 8    |
| 1.5 Biological Actions of IGFs                | 11   |
| 1.6 IGF Receptors                             | 12   |
| 1.7 (IGF)-High Affinity Binding Proteins      | 15   |
| 1.7.1 IGF-BP-1                                | 16   |
| 1.7.2 IGF-BP-2                                | 16   |
| 1.7.3 IGF-BP-3                                | 17   |
| 1.7.4 IGF-BP-4                                | 19   |
| 1.7.5 New IGF-BP                              | 19   |
| 1.8 Biological Actions of IGF-BPs             | 21   |

|  |           |
|--|-----------|
| <b>2. MATERIALS AND METHODS</b>  | <b>25</b> |
| 2.1 Animals  | 25        |
| 2.2 Materials and Chemicals  | 25        |
| 2.3 Preparation of Primary Hepatocyte Cultures   | 26        |
| 2.4 Hormonal Stimulation and Media Collection  | 28        |
| 2.5 Iodination of IGF-I  | 28        |
| 2.6 Charcoal Binding Assay for IGF-BPs   | 28        |
| 2.7 Sephadex G-75 Acid Gel Chromatography  | 29        |
| 2.8 Specificity of IGF-BPs   | 29        |
| 2.9 Western Blotting (WB) for IGF-BPs  | 30        |
| 2.9.1 Sample Preparation   | 30        |
| 2.9.2 Molecular weight Markers   | 31        |
| 2.9.3 SDS-PAGE Electrophoresis   | 31        |
| 2.9.4 Electroblotting  | 34        |
| 2.10 Immunoprecipitation of IGF-BPs Followed<br>by Western Blotting (WB)   | 36        |
| 2.11 Densitometry  | 37        |
| <b>3. RESULTS</b>  |           |
| 3.1 IGF-BP Secretion in Conditioned Media<br>Rat Hepatocyte Cultures   | 38        |
| 3.2 Western Blotting   | 43        |
| 3.3 Time-course of GH and/or Glucagon Effect on<br>IGF-BPs secreted by Adult Rat Hepatocytes<br>in Primary Culture | 47        |

|  |    |
|--|----|
| 3.4 IGF-BP Analysis by Gel Filtration Method                           | 47 |
| 3.5 Competitive Binding Studies of the Different<br>Species of IGF-BPs | 51 |
| 3.6 Immunoprecipitation of IGF-BPs Prior to<br>Western Ligand Blotting | 57 |
| 4. DISCUSSION AND CONCLUSION   | 61 |
| 4.1 IGF-Binding Activity   | 61 |
| 4.2 IGF-Binding Protein(s) Characterization                            | 62 |
| 4.3 Growth Hormone (GH) Effects on IGF-BPs                             | 66 |
| 4.4 Glucagon Effects on IGF-BPs  | 68 |
| 4.5 Combined Effects of GH and Glucagon<br>on IGF-BPs                  | 69 |
| 6. REFERENCES  | 71 |

## LIST OF FIGURES

| FIGURE | TITLE   | PAGE |
|--------|---|------|
| 1      | Diagram of the CNS-Hypothalamic-Pituitary Axis of Control   | 2    |
| 2      | Diagrammatic Representation of the Principal Mechanisms of GH control   | 5    |
| 3      | Amino Acid Sequence of Human IGF-I  | 9    |
| 4      | Amino Acid Sequence of Human IGF-II   | 10   |
| 5      | Schematic Comparison Between Type I and Type II IGF Receptors and the Insulin Receptor                                  | 14   |
| 6      | Diagrammatic representation of the Circulating IGF-BP complex   | 18   |
| 7      | Diagram of the Structural Characteristics of the four Known IGF-BPs in Human and Rat                                    | 20   |
| 8      | The IGF-I Binding Activity Secreted by Adult Hepatocytes in Culture   | 39   |
| 9      | Effect of bGH and/or Glucagon on the secretion of IGF-BPs by Cultured Rat Hepatocytes after 24 h of Incubation          | 40   |
| 10     | Effect of Lyophilization of media (10x 0.01 N HCl) on IGF binding to IGF-BPs Secreted by the Rat Hepatocytes in culture | 42   |
| 11     | Effect of Lyophilization of media (10x 0.01 N PBS) on IGF binding to IGF-BPs Secreted by the Rat Hepatocytes in culture | 42   |

|    |   |    |
|----|---|----|
| 12 | Western Blotting of the Different Species of IGF-BPs Secreted by the Rat Hepatocytes in Culture after 24 h of Incubation in Serum-free Medium                                 | 44 |
| 13 | Effects of bGH and/or Glucagon on the Different Species of IGF-BPs Secreted by the Cultured Hepatocytes after 24 h of Incubation  | 45 |
| 14 | Densitometric Scan of the Autoradiogram shown in fig. 15  | 46 |
| 15 | Time-dependent Effect of bGH and/or Glucagon on IGF-BPs Secreted by Rat Hepatocytes   | 48 |
| 16 | Analysis of IGF-BPs Secreted by the Cultured Rat Hepatocytes by Sephadex G-75 Acid Gel Chromatography   | 49 |
| 17 | Analysis of IGF-BPs Secreted by the Cultured Rat Hepatocytes by Sephadex G-75 Acid Gel Chromatography   | 50 |
| 18 | Dose-dependent Inhibition of $^{125}\text{I}$ -IGF-I to Acid-stripped Binding Proteins from Cultured Adult Rat Hepatocytes  | 52 |
| 19 | Dose-dependent Inhibition of $^{125}\text{I}$ -IGF-I to Acid Stripped Binding Proteins from Cultured Adult Rat Hepatocytes  | 53 |
| 20 | Dose-dependent Inhibition of $^{125}\text{I}$ -IGF-I and $^{125}\text{I}$ -IGF-II Binding to Acid Stripped Binding Proteins by Different Concentrations of Unlabeled Peptides | 54 |
| 21 | Scatchard Analysis of Dose-dependent Inhibition Data obtained from IGF-BPs secreted by adult rat hepatocytes  | 55 |

|    |  |           |
|----|--|-----------|
| 22 | <b>Immunoprecipitation of Media from Adult Rat Hepatocytes using Antiserum to rIGF-BP-1 and rIGF-BP-2 Prior to Ligand Blotting</b> | <b>58</b> |
| 23 | <b>Densitometric Scan of Immunoprecipitated IGF-BPs Present in the Media from Adult Rat Hepatocytes</b>                            | <b>60</b> |

## LIST OF TABLES

| TABLE | TITLE  | PAGE |
|-------|--|------|
| 1     | Sample Buffer Composition  | 30   |
| 2     | Resolving Gel Composition  | 32   |
| 3     | Stacking Gel Composition   | 33   |
| 4     | Electrophoresis Buffer   | 34   |
| 5     | Transfer Buffer (TB)   | 34   |
| 6     | The Effect of bGH and/or Glucagon on<br>IGF-BP accumulation in media from<br>Adult Rat Hepatocytes | 41   |
| 7     | Data on bGH and/or Glucagon's Effect<br>on BP secretion by the Hepatocytes in<br>Culture           | 48   |
| 8     | Scatchard Analysis of IGF-BPs from<br>media of Adult Rat Hepatocytes                               | 56   |
| 9     | Densitometric Data from Autoradiogram<br>shown in fig. 22  | 59   |

# CHAPTER 1: INTRODUCTION

## 1.1 The Neuroendocrine Axis of Control

It was not until the beginning of this century that the hypothalamus was recognized to play an important role in endocrine, behavioral, and autonomic adjustments of an organism. The hypothalamus contains centers that regulate temperature, blood pressure, heart rate, respiration, sexual behavior, reproduction, emotions, food and water intake, and most biological rhythms. The endocrine and nervous systems are interactive; and it is believed that the hypothalamus is the organ that mediates these interactions. Neuroendocrine control is centrally rather than peripherally maintained through the following axis:

CNS (central nervous system) --> Hypothalamus --> Pituitary --> target gland  
(Fig. 1).

There are two types of hypothalamic neurons :

a) the large magnocellular neurons which are primarily involved in synthesis, transport, and release of the hormones oxytocin and vasopressin (anti-diuretic hormone, ADH).

b) the smaller parvicellular neurons originating from the hypothalamus which are involved in the synthesis and release of factors containing hypothalamic information (releasing hormones). These factors reach the anterior pituitary lobe by the hypophyseal portal circulation and stimulate or inhibit the synthesis and/or secretion of anterior pituitary hormones. The principal factors described to date are: gonadotropin releasing hormone (GnRH), corticotropin releasing hormone (CRH), thyrotropin releasing hormone (TRH), somatostatin (SS), and growth hormone releasing hormone (GHRH). These factors regulate the secretion of luteinizing

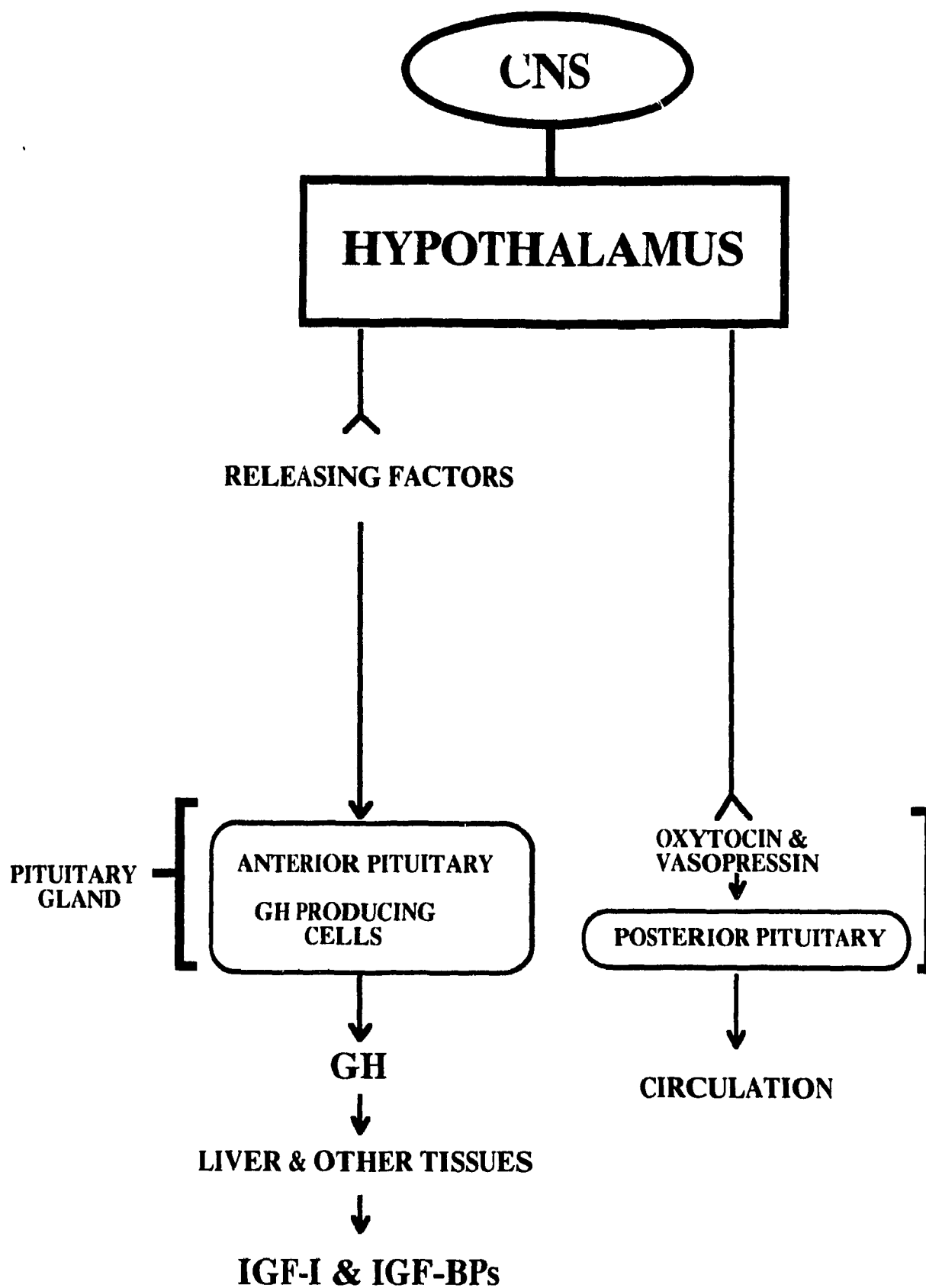


Fig. (1). Diagrammatic representation of the CNS-Hypothalamic-Pituitary axis of control.

hormone (LH), follicle stimulating hormone (FSH), corticotropin releasing hormone (CTH), thyroid stimulating hormone (TSH), growth hormone (GH) and prolactin (PRL).

(GHRH) and (SS) act on the anterior pituitary somatotrophic cells to regulate GH production and secretion in the circulation. Circulating growth hormone in turn, controls the circulating levels of insulin-like growth-factor -I (also referred as Somatomedin-C) produced in the liver.

## **1.2 Regulation of Growth Hormone Secretion**

The anterior part of the pituitary gland is made up of various epithelial cells which have secretory functions. These secretory functions are under strict control from hypothalamic releasing factors. Cells have been classified according to staining reactions (Purves et al., 1966) and divided into three groups:

- a) acidophils , positive reaction with acidic dyes
- b) basophils , positive reaction with basic dyes
- c) chromophobes which do not stain with either dye system

GH producing cells (somatotrophs) have been identified as acidophilic and GHRH was found to bind specifically to their cell surface (Seifert et al., 1985; Zysk et al., 1986). Hypothalamic GHRH is a 44 amino acid peptide with an amidated carboxy terminal region (Gelato et al., 1986). The physiological action of this peptide is very specific and directly involves the stimulation of GH secretion from the somatotrophic cells of the anterior pituitary (Guillemin et al., 1982). It also increases pituitary GH mRNA at the transcription level (Fucata et al., 1985; Barringa et al., 1983; Gick et al., 1984). Pituitary growth hormone stimulation occurs in a dose-dependent fashion with maximal GHRH stimulation effected by a concentration of 100 pmol (Vale et al., 1983).

A potent inhibitor of GH release is somatostatin (SS). There are two biologically active forms of SS, the SS-14 and SS-28 which both originate from a large precursor (preproSS). Somatostatin receptors are found at the pituitary level (Srikant et al., 1982; Aguilera et al., 1982), in brain synaptosomal membranes (Srikant et al., 1981), and other various tissues. More recently there is evidence of receptor subtypes (Srikant et al., 1986) linked with multiple signal transducing systems. The inhibition of GH release from secretory granules in somatotrophs is triggered by SS binding to its high affinity receptor followed by Gi-protein signal transduction (Bourgoyne, 1987). The neurohormonal mechanisms involved in regulating growth hormone secretion are complex. In addition to the above hypophysiotropic hormones there are neurotransmitter mechanisms originating from the central nervous system (Fig.2).

### **1.3 Growth Hormone and IGFs**

Growth hormone (GH) is a large single chain polypeptide hormone synthesized and secreted by acidophilic cells located in the anterior pituitary gland (Isaksson et al., 1985). GH is very similar in structure to other two hormones, prolactin and placental lactogen. All these three hormones in man possess lactogenic activities (Collu, 1989); however only GH is growth promoting. GH consists of 191 amino acids with a large central disulfide loop between Cys<sup>53</sup> and Cys<sup>165</sup> and a small loop between Cys<sup>182</sup> and Cys<sup>189</sup>. GH increases the size of the skeleton, the muscles, and the connective tissue mass. It also has direct effects on carbohydrate and lipid metabolism (Isaksson et al., 1985). GH receptors have been found in a variety of tissues such as adipocytes, liver and fibroblasts (Mendelsohn, 1988). There is also evidence for GH receptor subtypes (Hughes et al., 1985). The molecular weights of GH receptors have been estimated to be between 50-110 kDa (kilodaltons) by SDS-PAGE depending on the source of isolation (Hughes et al., 1985; Mathews et al., 1989; Hughes and Friesen, 1985).

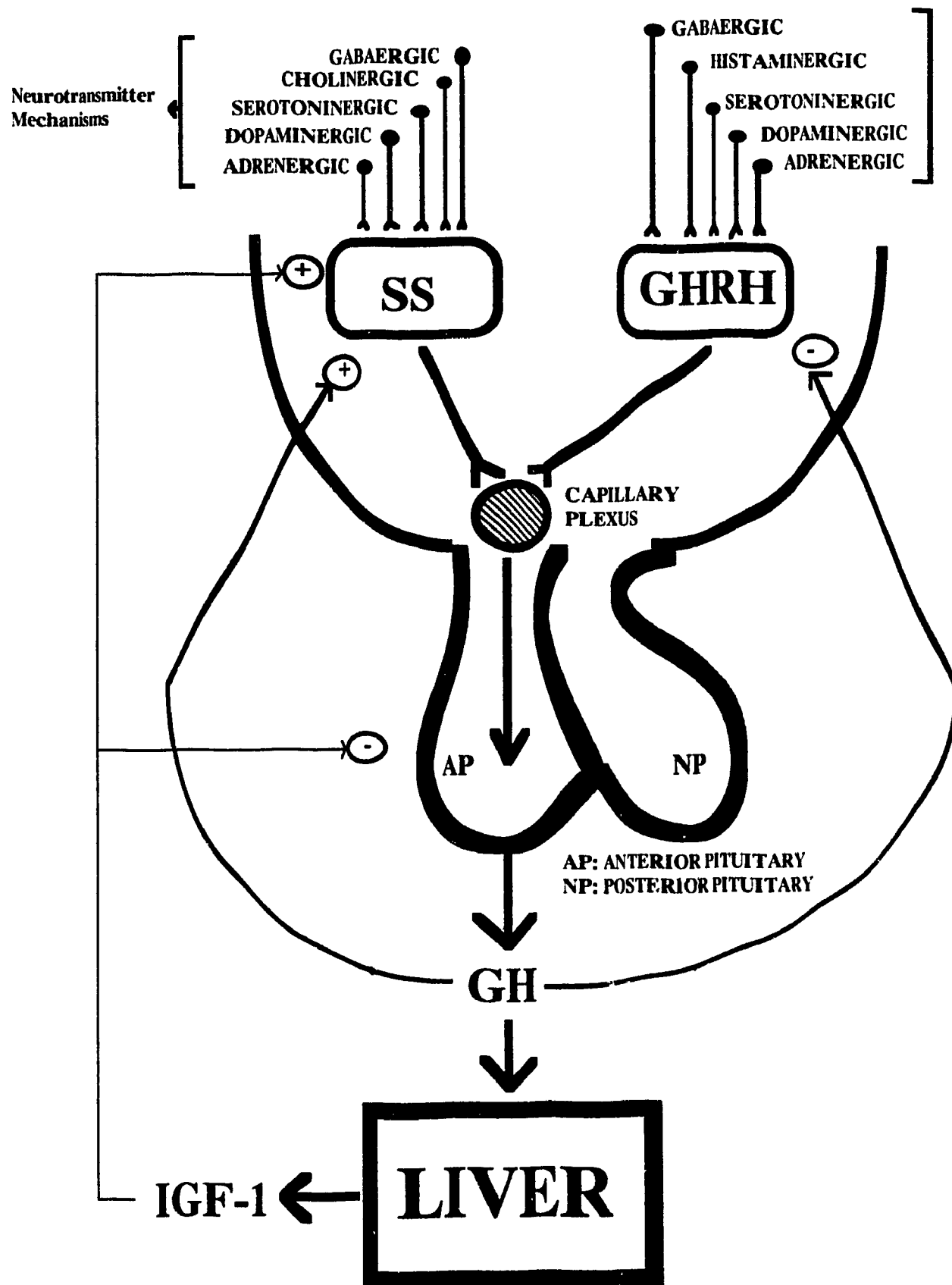


Fig. (2). Diagrammatic representation of the principal mechanisms of GH control.

When GH binds to its high affinity receptor in target organ(s), growth effects may not always be direct. Early studies of hypophysectomized rats showed a defect in the synthesis of cartilage matrix proteins. GH administration *in vivo* rapidly repaired this defect. Parallel *in vitro* studies demonstrated that growth hormone failed to increase incorporation of radiolabeled sulfate [ $^{35}\text{SO}_4^{-2}$ ] into the chondroitin sulfate component of cartilage. Addition of dilute normal rat serum did increase [ $^{35}\text{SO}_4^{-2}$ ] incorporation (Salmon and Daughaday, 1957) whereas hypophysectomized rat serum had no effect. Therefore an intermediary agent present along with GH in normal serum seems to stimulate sulfate incorporation into cartilage.

It became evident from these experiments that GH did not exercise a direct effect on skeletal tissue but, rather, acted through an intermediate substance. The concentration of this substance in the blood was demonstrated to be under the direct positive influence of circulating GH levels. This substance was initially named after its function "sulfation factor" and was subsequently termed somatomedin (Daughaday et al., 1972). It is now widely referred to insulin-like growth factor (IGF). Several investigators reported different somatomedins or IGFs which had similar characteristics. Initially they were known as somatomedin-A (SM-A), somatomedin-B (SM-B), and somatomedin-C (SM-C). With increasing knowledge of their structures SM-C was found to be identical to IGF-I (Enberg et al., 1984), SM-A was resolved into two components (a large IGF-I like and a small IGF-II like components) (Klapper et al., 1983), and SM-B was abandoned since epidermal growth factor (EGF) was the main contaminant found to give insulin-like activities (Heldin et al., 1981).

IGFs also possess insulin-like activities. During the early 60's, a non-suppressible insulin-like activity (NSILA) whose biologic action persisted in the presence of excess insulin antibodies was shown to stimulate rat adipose tissue (Leonards et al., 1962). It was soon observed that NSILA's constituted more than 90% the total serum insulin-like activity (ILA) (Jakob et al., 1968). In 1966, Bürgi et al. reported a partial purification of NSILA by acid-ethanol extraction from human plasma. Ten years later, Rinderknecht and Humbel (1976a)

resolved NSILA into two distinct components, NSILA-I and -II whose structures were determined. They were renamed insulin-like growth factor I (IGF-I) and IGF-II respectively.

In the early 70's, IGFs were also identified from rats. Molecules possessing cell multiplication stimulating activity were first isolated from rat source by Dulak and Temin, 1973. These macromolecules showed insulin-like metabolic activities, sulfate incorporation into cartilage, and stimulated chick embryo fibroblast division. From the assay used, they were named "multiplication stimulating activity" or MSA. After complete purification (Moses et al., 1980), MSA was resolved into several different molecular weight species of 16.3 , 8.7 , and 7.1 kilodaltons (kDa). These investigators reported that all the MSA species represented fragments of a large precursor. When the complete sequence of the 8.7 kDa fragment was obtained, it was found to be similar to human IGF-II (62 of 67 amino acids were identical) (Marquardt et al., 1981).

Thus a range of studies revealed the existence of a new family of peptides known as IGFs. It is now established that: (i) the concentration of IGFs in the serum is growth hormone dependent, (ii) they show insulin-like effects in a variety of tissues, and (iii) they are able to stimulate a range of anabolic activities including sulfate incorporation into cartilage.

The relation between GH and IGFs was viewed as involving GH action on target tissues via GH receptors, triggering the biosynthesis and/or secretion of insulin-like growth factors (mainly IGF-I). IGF-I released into circulation can subsequently act on peripheral tissues to restore growth and/or metabolism. Adding to this putative endocrine role of IGFs, is the recent appreciation that the IGFs have paracrine and perhaps autocrine roles. Thus the main mediator of the growth promoting actions of GH may be IGF-I. At the molecular level, the mechanism of action of GH is largely unknown. This was due to the difficulties in demonstrating GH effects *in vitro* on known target tissues. However, in our laboratory we have established a method for studying GH effects *in vitro* and found that GH seems to act in

conjunction with a cAMP-dependent pathway (Kachra et al., unpublished results). It is still unclear whether IGF-I alone can mediate all the anabolic effects of GH. The fact that all tissues express IGF-I (Mathews et al., 1986; Han et al., 1987), and that GH receptors are also found in a wide variety of tissues, including liver (Barnard et al., 1988), supports the view that GH and IGF-I may operate in conjunction with one another. Barnard's group found that epiphyseal chondrocyte growth was stimulated by the local administration of GH and not by IGF-I. This suggests a role for both GH and IGF-I in differentiation and growth (Green et al., 1985). It is also known that GH binding to its receptor leads to a phosphorylation of tyrosine (Tyr) residues on the C-terminal cytoplasmic region of the receptor (Foster et al., 1988). The phosphorylated receptor is unusual because it lacks intrinsic tyrosine kinase activity. To clarify the mechanism of action of GH, IGFs and their participating roles with GH are being studied intensively.

#### **1.4 Insulin-like Growth Factors (IGFs)**

Insulin-like growth factors (IGFs) are important multifunctional polypeptides. They comprise a family of peptides which promote cell differentiation and proliferation, as well as insulin-like metabolic effects. Rinderknecht and Humbel, in 1976, resolved the IGFs into IGF-I and IGF-II. Both IGF-I and IGF-II are single chain polypeptides composed of 70 and 67 amino acids with molecular weights of 7649 and 7471 daltons, respectively (Rinderknecht and Humbel, 1978). Each single-chain peptide consists of domains B, C, A and D; in which A and B are structural homologs of the insulin A and B chains (see Figs. 3 and 4). Domain C is analogous to the connecting (C) peptide in pro-insulin, whereas the D domain is not found in insulin (Humbel, 1984). The degree of homology between these peptides is approximately 62% and both are highly homologous to proinsulin and relaxin. The three-dimensional structure of the IGFs has not been determined, but computer-assisted molecular modeling has simulated 3-D structures analogous to proinsulin. Theoretical predictions for binding of IGFs to receptors or high affinity binding proteins have been made and now can be tested with powerful techniques such as site-directed mutagenesis. The concentration of IGFs in blood plasma is between 20-



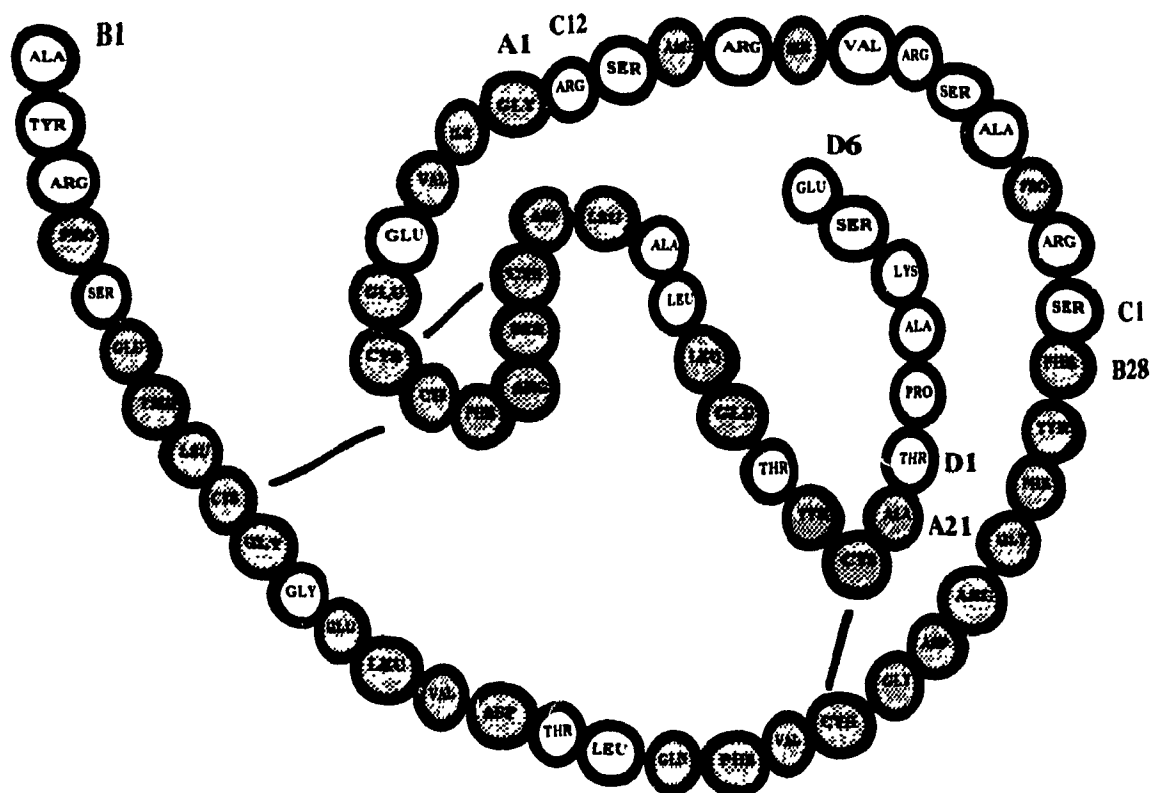


Fig. (4). Amino acid sequence of human IGF-II. The amino acids which are identical to those found in IGF-I are shaded. IGF-II consists of four domains (B, C, A, and D) with 28, 12, 21, and 6 amino acids respectively. Rat IGF-II is highly homologous except for amino acids B22 (GLY  $\rightarrow$  SER), C4 (ALA  $\rightarrow$  SER), C7 (VAL  $\rightarrow$  SER), and C8 (SER  $\rightarrow$  ASN). Black lines represent disulfide bridges between cysteine residues.

80nM whereas in a variety of tissues the concentration is much lower and sometimes non-detectable with radioimmunoassays.

The major organ that synthesizes and secretes IGFs into the circulation is the liver. Extensive research is continuing to distinguish between endocrine and paracrine/autocrine roles for the IGFs.

### **1.5 Biological Actions of IGFs**

IGF-I appears to act primarily as a regulator of postnatal growth and is under strict GH regulation. IGF-II's synthesis is less tightly controlled by GH than IGF-I and the physiological role(s) of this peptide is far from being elucidated.

It has been demonstrated that IGFs promote growth *in vivo* (Schoenle et al., 1985). These investigators showed dose-dependent IGF-I stimulation of growth in hypophysectomized rats. When comparing potency for growth stimulation between the two peptides, IGF-I was more potent than IGF-II. In another study, GH stimulated bone growth in cultured osteoblast-like cells but when antibodies directed against IGF-I were co-incubated with GH, the growth effects were abolished (Ernst and Froesch, 1988). Additionally when GH and IGF-I were added simultaneously, an additive effect was reported (Ernst and Froesch, 1988). Therefore these authors showed that IGF can act in a paracrine and autocrine fashion. After careful analysis of the accumulated data on IGF action, it was concluded that GH stimulates local production of IGFs but the overall cell proliferation action of GH is the predominant mechanism for skeletal growth (Daughaday, 1989). Scheiwiller et al. (1986) showed that IGF-I restored growth in insulin-deficient diabetic rats. Therefore these authors proposed that the growth promoting actions are mediated through IGF-I at the cellular level whereas GH and insulin regulate the hepatic synthesis and availability of IGF-I.

The original somatomedin hypothesis was modified because IGF-I was not able to replace GH and indeed GH is the essential local inducer of IGF-I. Humbel suggested that the growth promoting activity of GH is partly due to the ability of tissues to respond to IGF-I and partly to indirect effects on liver IGF-I production to increase serum concentrations of this peptide. Also IGF-I and GH act synergistically on target tissues. It should not be forgotten that IGF-I cannot mimic all the effects of GH.

Therefore the concentration of free IGF-I in plasma may play a role in GH-regulated mitogenesis, cell proliferation, and overall somatic growth. It is well accepted that IGFs bind and act through their specific cell surface receptors. To further complicate the IGF field, there are specific high affinity binding proteins in blood plasma which bind to IGFs with equal affinity to that of their receptors. These binding proteins have been purified from plasma and other tissues both in animals and man. Since many tissues synthesize their own IGFs and IGF-binding proteins, more emphasis is directed on the different binding proteins since they appear to regulate IGF action by controlling the availability of free IGFs and more importantly prolong the half-life of these peptides.

## **1.6 IGF Receptors**

IGF-I and insulin are homologous peptides which elicit two types of biological responses: a) a long term effect on cellular proliferation or b) a shorter term metabolic effect such as increased glucose transport. The biological actions of IGF-I and IGF-II are mediated by specific cell membrane receptors. These two receptors are distinct in structure, and in relative affinities for their respective ligands and are referred as types I and II. We will focus on the IGF-I and its type I receptor since the functions of IGF-II and its type 2 receptor remain less clear.

The type 1 IGF receptor has structural similarities to the insulin receptor (Fig. 5). They are both glycosylated heterotetramers composed of 2  $\beta$  subunits and 2  $\alpha$  subunits of 90 and 135 kDa respectively (Chernauser et al., 1981; Massague and Czeck, 1982). The extracellular part of the receptor consists primarily of  $\alpha$  subunits which contain the IGF-I binding site(s). The  $\beta$  subunits are intrinsic membrane proteins with a transmembrane hydrophobic domain and an intracellular tyrosine kinase domain (Rubin et al., 1983). cDNAs obtained for insulin receptor (Ullrich et al., 1985) and the type I IGF receptor (Ullrich et al., 1986) revealed a high degree of homology between the two receptors with regard to amino acid sequence, and subunit size and structure. A very interesting observation was the degree of homology in the tyrosine kinase domain (84% identity). Once the IGF-I molecule binds to its receptor, there is a rapid internalization and activation via tyrosine autophosphorylation of the  $\beta$  subunit (Jackobs et al., 1983). In addition phosphorylation occurs at serine and threonine residues of the  $\beta$  subunits since serine and threonine kinase(s) are also activated (Czech, 1989). Following tyrosine kinase activation, there is a cascade of events involving phosphorylation-dephosphorylation reactions leading to the biologic effect which in overall terms results in cell division.

The IGF-II receptor is structurally distinct from the IGF-I receptor. It is a monomer of approximate molecular weight of 250 kDa and does not possess autokinase activity but can act as a substrate for a membrane-associated tyrosine kinases (Covera et al., 1986). cDNA for the type II IGF receptor revealed similarities to the bovine cation-independent mannose-6-phosphate receptor, a receptor capable of lysosomal enzyme transport (Morgan et al., 1987). From the receptor sequence, 92% of the receptor is extracellular (Fig. 5).

For most cells, type I and type II receptors are present at the cell surface. To further complicate the puzzle, the insulin receptor is found to coexist in certain cell lines with both IGF receptors. This increases the difficulty in establishing exact binding affinity constants for each ligand to their respective high affinity receptor. However, through experiments performed on cell lines engineered to preferentially express one type of receptor, there is evidence that

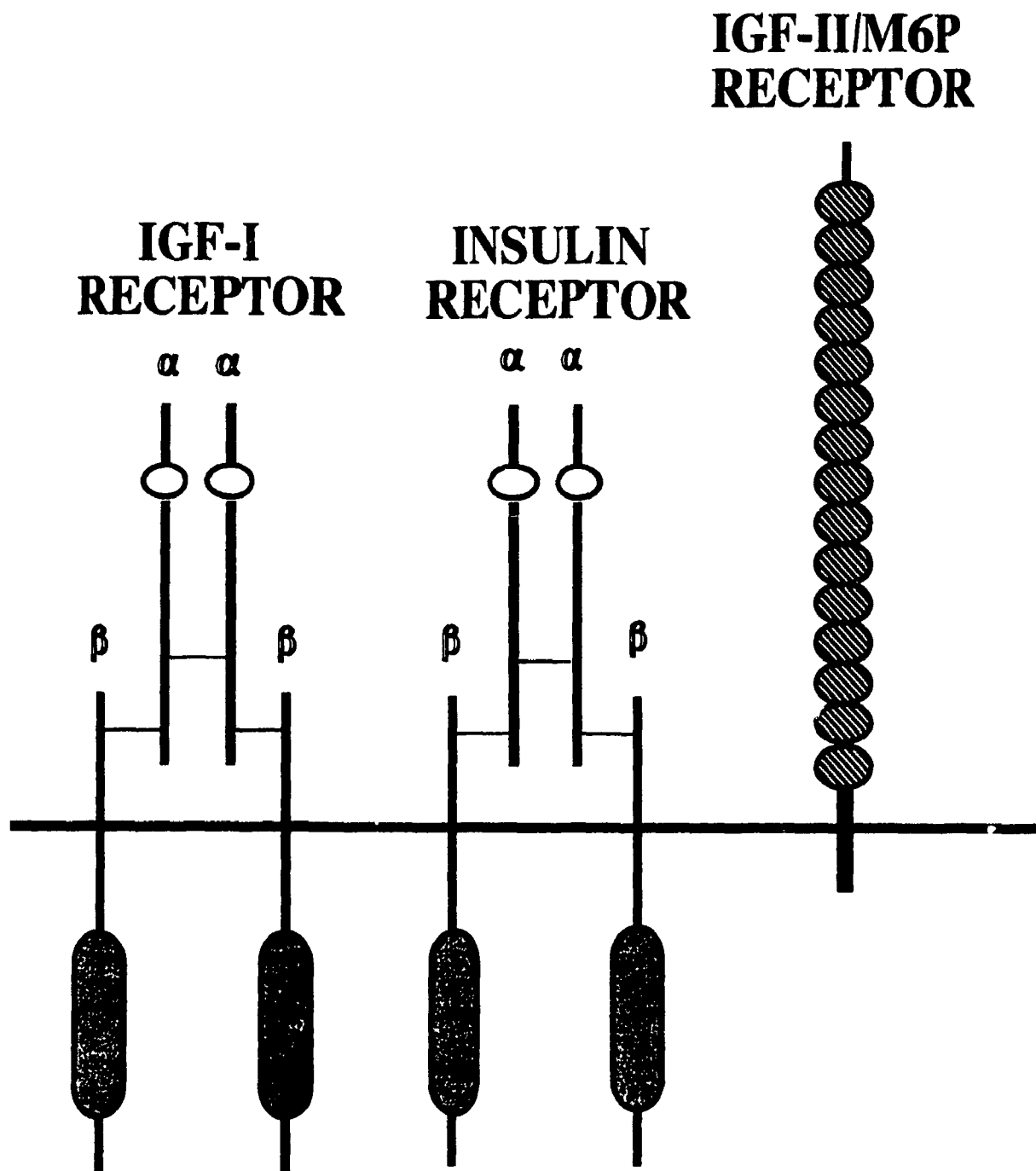


Fig. (5). Schematic comparison between type I and II IGF receptors and the insulin receptor. ( Abbreviation: IGF-II/M6P, IGF-II or cation dependent manonose-6-phosphate receptor). Open circles are ligand binding sites and shadowed areas are tyrosine kinase domains. The circles on the IGF-II/M6P receptor represent repeated extracellular cysteine-rich regions.

heterologous ligands (eg. insulin) have lower affinity towards heterologous receptors (eg. type I IGF receptor) and vice versa (Megyesi et al., 1974). There is substantial evidence (Baxter and Martin, 1989) that the relative affinities of ligand-receptor complexes varies according to specificity. It is well accepted that: i) type I IGF receptor binds IGF > IGF-II » insulin, ii) type II IGF receptor binds IGF-II > IGF-I (no insulin binding), and iii) the insulin receptor binds insulin » IGF-II > IGF-I.

### **1.7 IGF-High Affinity Binding Proteins (IGF-BPs)**

The IGFs in serum and other biological fluids are complexed to specific binding proteins, IGF-BPs (Nissley and Rechler, 1984). The mechanism of release of these binding proteins is not known at present. The N-terminal region of IGFs (first three amino acids) are crucial for interactions with BPs (Ross et al., 1989). Ongoing research is aimed at elucidating the endocrine vs. autocrine/paracrine roles of these growth factors in conjunction with IGF-binding proteins. Circulating IGFs may play an endocrine role in influencing tissue function(s) and in coordinating growth.

A major development in this field occurred when cloning and sequencing of these different IGF-BPs was performed in various laboratories around the world. Currently, there are five different species of IGF-BPs identified in humans and rats. Up to date only four IGF-BP species have been cloned and characterized extensively. These binding proteins show large homology from species to species. The classification process is based both on immunoreactivity and amino acid sequence. More information about their complex structures should be an asset for understanding more about their role(s) in regulating metabolic and mitogenic events.

### 1.7.1 IGF-BP-1

IGF-BP-1 was detected in amniotic fluid (Chochinov et al., 1977). This acid-stable protein showed a molecular weight of 35-40 kDa and was further characterized by several investigators (Drop et al., 1979; Povia et al., 1984a; Baxter et al., 1987). Baxter generated polyclonal antibodies against this IGF-BP and subsequently used these antibodies to characterize other BPs secreted by different tissue. Before its purification from amniotic fluid, the protein had been purified from fetal extracts and maternal placenta (named placental protein 12 = PP12) (Kiostinen et al., 1986).

Binding proteins from various sources were characterized and found to belong to the family of IGF-BP-1 such as: (a) an IGF-BP found in the conditioned media of a human hepatoma cell line, HEP G2 (Moses et al., 1983) ; and (b) a pregnancy-associated endometrial  $\alpha_1$ -globulin ( $\alpha_1$ -PEG) (Bell et al., 1986; 1988; 1988a).

Rat IGF-BP-1 has 72% homology with the human IGF-BP-1. The predicted molecular weights based on the mRNA sizes are 25,274 and 29,600 kilodaltons (kDa) respectively (Lee et al., 1988; Murphy et al., 1990). Molecular weights have been found to vary from 25-40 kDa depending on conditions (reducing vs. non-reducing) and on the method used (ultracentrifugation, SDS-PAGE). Although molecular weight determinations from DNA sequences are more accurate than any other method, they do not account for post-translational modifications (viz. glycosylation), etc..

### 1.7.2 IGF-BP-2

IGF-BP-2 has been found in human CSF (Hossenlopp et al., 1986; Rosenfeld et al., 1989), in rat BRL-3A cells (Moses et al., 1979) and in bovine kidney (MDBK) cells (Szabo et al., 1988). These binding proteins did not react with IGF-BP-1 antibodies.

The cDNA for BP-2 has been isolated from human and rat species. In humans, the mature form contains 289 amino acid residues which differs from the rat's 270 amino acid residues. However from rat and human cDNAs (Brown et al., 1989; Margot et al., 1989), there was 83% homology between species (Binkert et al., 1989) with predicted molecular weights of 29,564 and 31,300 kilodaltons (kDa) respectively. IGF-BP-2 contains an Arg-Gly-Asp sequence (sequence for potential cell anchoring character) near the carboxy-terminal region. The homology between IGF-BP-1 and IGF-BP-2 is less than 40%.

In the rat, IGF-BP-2 has been immunologically detected in a variety of fetal tissues which emphasized the fetal predominance of this protein (Brown et al., 1989). Although IGF-BP-2 has been thought to be involved in developmental regulation, there are no specific known regulators. This binding protein has higher affinity for IGF-II than IGF-I.

### **1.7.3 IGF-BP-3**

Most of the IGFs in the plasma are bound to carrier proteins. It is known that Sephadex G-200 acid chromatography of plasma prior to incubation with  $^{125}\text{I}$ -IGF-I yields IGF binding activity in both 40 and 150 kDa elution regions (Hintz and Liu, 1980). The 150 kDa complex is composed of three subunits, as seen in Fig. 6 below, where the  $\beta$ -subunit of IGF-BP-3 is the IGF binding subunit (Baxter and Martin, 1989).

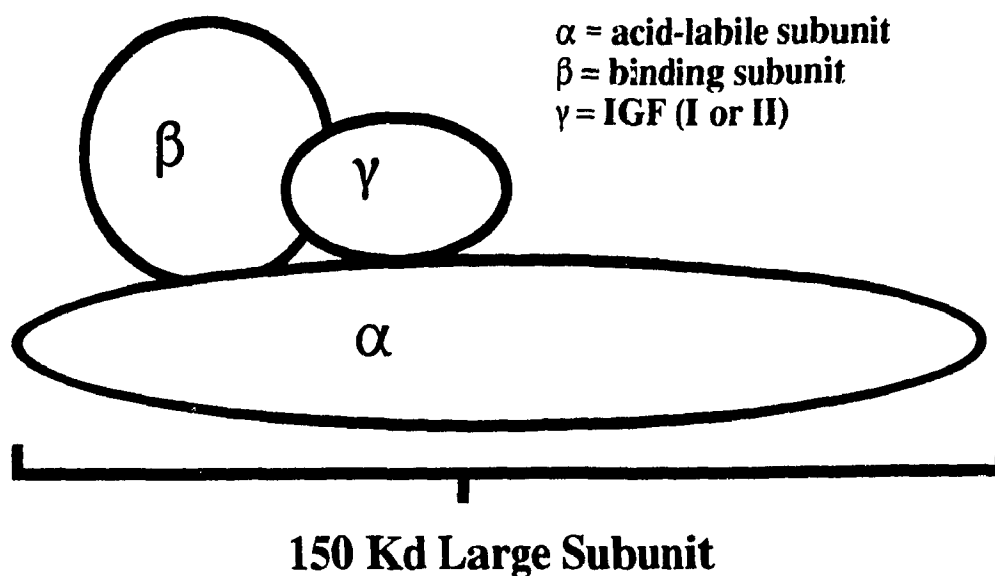


Fig. (6). Diagrammatic representation of the circulating IGF-BP complex.

IGF-BP-3 is an acid-stable glycoprotein. From SDS-polyacrylamide gel electrophoresis analysis, as of adult human serum, it appears as a major and a minor band of  $M_r$  = 53,000 and 47,000 kilodaltons respectively (Baxter and Martin, 1989).

After treatment with a deglycosylation enzyme (deglycosylase endoglycosidase = Endo F), both bands are reduced in molecular weight into one band with an  $M_r$  = 29,000 kDa. This suggested different degrees of glycosylation (Lamson et al., 1989)

The other components of the large molecular weight complex are acid-labile. The  $\alpha$ -subunit does not bind to IGF-I or IGF-II, and has a molecular weight of 84-86 kDa.

cDNA of human IGF-BP-3 revealed three asparagine residues as potential N-glycosylation sites, and an estimated molecular weight of 28,500 kDa (Wood et al., 1989). This binding protein showed 33% homology with IGF-BP-1 but lacked the C-terminal Arg-Gly-Asp sequence present in both IGF-BP-1 and -2 (Wood et al., 1988). Like IGF-I, IGF-BP-3 is GH-

dependent and circulates with concentrations mirroring those of IGF-I. This suggests that serum BP-3 is fully saturated with ligand.

#### **1.7.4 IGF-BP-4**

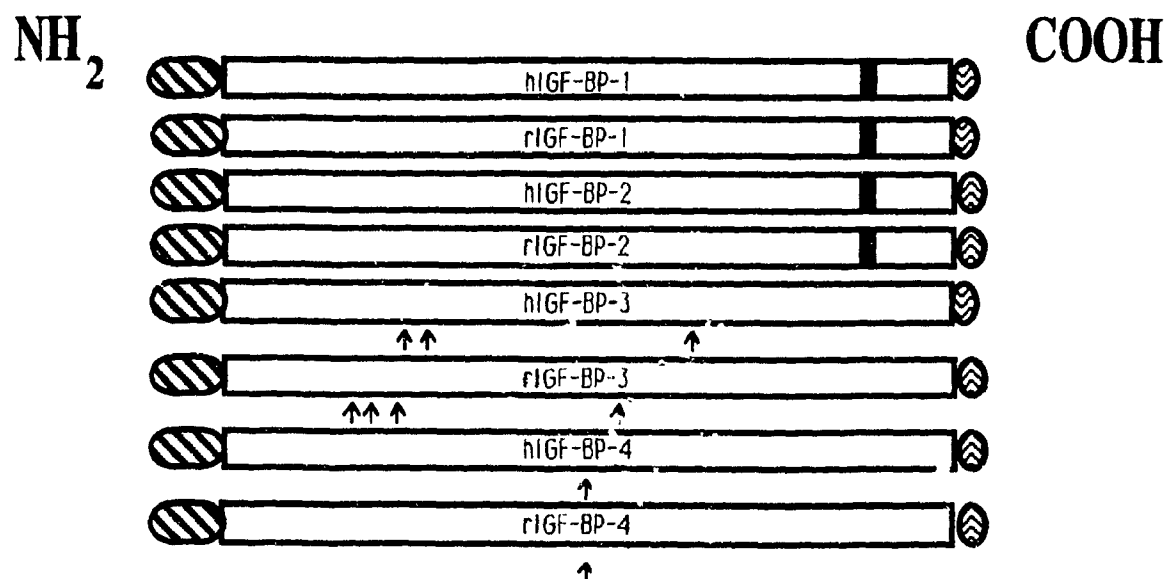
Antibodies against IGF-BPs -1, -2, or -3 did not recognize this new low molecular weight binding protein identified in human and rat serum (Donovan et al., 1989).

The estimated molecular weight from the complementary DNA of human placenta is 25,970 kDa (Shimasaki et al., 1990). A similar-sized IGF-BP was found in the media from human fibroblasts (Conover et al., 1989), human breast cancer cells (De Leon et al., 1989), and seminal fluid (Rosenfeld et al., 1989b). This BP was initially believed to be a degradation product of IGF-BP-3 until antibodies against IGF-BPs -1, -2, and -3 showed no crossreactivity.

#### **1.7.5 NEW IGF-BP**

This IGF-BP has not been fully characterized yet. The purified protein has a different N-terminal region compared to IGF-BPs 1 to 4. Human CSF is the source of this fifth type of IGF-BP (Roghani et al., 1989).

The structural characteristics of the IGF-BPs that have been sequenced up to date are summarized in Fig.7. There are regions where the protein structure of the various binding proteins is well conserved. For example, cysteine rich regions are found in both amino and carboxy-terminal regions throughout the four binding proteins. Also the positions of the Cys residues are well conserved which suggest similar secondary protein structures. In rat and



| IGF-BP TYPE | NUMBER OF<br>AMINO ACIDS | MOLECULAR<br>WEIGHT (kDa) | NUMBER OF<br>CYSTEINES |
|-------------|--------------------------|---------------------------|------------------------|
| hIGF-BP-1   | 234                      | 25,274                    | 18                     |
| rIGF-BP-1   | 247                      | 29,600                    | 18                     |
| hIGF-BP-2   | 289                      | 31,300                    | 20                     |
| rIGF-BP-2   | 270                      | 29,564                    | 18                     |
| hIGF-BP-3   | 264                      | 28,500                    | 18                     |
| rIGF-BP-3   | 265                      | 28,900                    | 18                     |
| hIGF-BP-4   | 237                      | 25,970                    | 20                     |
| rIGF-BP-4   | 233                      | 25,700                    | 20                     |

Fig.(7). Diagrammatic representation showing structural characteristics of the four known IGF-BPs in human and rat. The diagrammatic bars do not accurately represent the number of amino acids per peptide. Potential asparagine N-linkage points for glycosylation (arrows) and the Arg-Gly-Asp (RGD) sequences (vertical solid lines) as indicated. Human and rat IGF-BP-4 have 2 cysteines that are not conserved within the other species. Molecular weights are predicted from cDNA's.

human IGF-BP-4, there are two extra cysteine residues located in the middle of the protein that are not conserved in other BPs. This may suggest that these smaller molecular weight BPs have different secondary structure. This difference in secondary structures within the binding proteins may alter IGF binding capacities, the biological significance of which remains to be determined. An important feature revealed from amino acid sequences of BP's is the conserved C-terminal Arg-Gly-Asp (RGD) sequence in IGF-BP-1 and -2 only. The conserved sequences are found in secretory protein families which have cell surface anchoring properties (Ruoslahti and Pierschbacher, 1987). Therefore both IGF-BP-1 and -2 may be bound to cell surfaces unlike IGF-BP-3 which lacks the conserved amino acid sequence.

It is interesting to note some relevant differences between IGF-BP-1, -2, -3, and 4. The position and number of glycosylation sites (N-linkage asparagine sites) in rat and human IGF-BP-3 and -4 differs. Protein glycosylation is one of the post-translational modification events that takes place before a mature IGF-BP is secreted. The function of the various N-glycosylation states is unknown, but the data indicate the presence of different populations of BPs in different species. Evidence for proteolytic cleavage of BPs as a post-translational modification event came from initial observations in pregnancy where IGF-BP-3 was cleaved into smaller subunits with decreased IGF affinity.

In general, IGF-BPs comprise a family of proteins which have similarities in both structure and function, and have been well conserved during the evolutionary process.

## **1.8 Biological Actions of IGF-BPs**

The mechanism of IGF-binding and the release of IGF from IGF-BPs for interaction with respective cell surface IGF receptors is not known. Initially the IGFs and IGF-BPs were thought to be synthesized and released from liver. After their release, the IGF-[IGF-BP]

complex could transport the IGF to the site of action. Therefore the major role of IGF-BP action could be to act as IGF-carrier molecules and prevent their degradation to ensure sufficient IGF supply to target tissues. Drakenberg et al. in 1990 showed that truncated IGFs had a lower affinity to IGF-BPs and interestingly a higher IGF biological activity.

In more recent studies, there is increasing evidence that both IGFs and some species of IGF-BPs are synthesized and secreted by multiple tissues. This adds a new dimension to IGF-BP action which emphasizes their local roles as modulators of IGF action.

Under normal physiological conditions, the serum concentration of insulin is 1000-fold lower than IGFs. The overall insulin-like activity due to IGFs is less than 5% that of insulin, therefore if the total serum IGF concentration were able to exert insulin-like effects, the symptoms would be as severe as in the hyperinsulinemic patient. Therefore the large molecular weight complex found in the circulation has inhibitory properties and limits excessive IGF action by regulating free IGF levels. However, when purified IGF-BP-3 was added to human skin fibroblast culture prior to IGF-I, there was an *in vitro* stimulatory IGF action (De Mellow and Baxter, 1988). But when co-incubated with IGF-I, cell-proliferation was inhibited. The functional role of the large 150 kDa complex from an endocrine view is getting more complex since many tissues preferentially express different binding protein(s). To better understand the involvement of peripherally synthesized IGF-BPs with IGFs, carrier IGF-BPs, and cell-surface receptors, their structure, regulation and elucidation of the functions of the different BPs is an asset.

Important information on the availability and the levels of specific IGF-BPs in the blood plasma came from studies performed on animals and patients in the normal versus diseased state. Much of today's understanding of the roles of IGFs and IGF-BPs in growth and metabolism has been deduced by comparing normal animals to animals in the fasted, diabetic, and hypophysectomized states.

There was initial evidence that circulating IGF bioactivity was reduced in animals (Phillips and Unterman, 1984; Eigenmann et al., 1977) and in patients (Winter et al., 1979; Samaan et al., 1963) with diabetes mellitus. More recent studies showed that the decrease in IGF bioactivity was due to an increase of circulating low molecular weight IGF-BPs and to a decrease in high molecular weight carrier protein complex (Unterman et al., 1990). Interestingly, the restoration of anabolism and promotion of protein synthesis by insulin administration decreased the level of the low molecular weight (32-34 kDa) IGF-BPs and increased the level of the high molecular weight (40-55 kDa) IGF-BP (Unterman et al., 1990). Therefore the modulation of tissue growth bioactivity in the diabetic diseased state may occur through insulin's regulation of low molecular weight IGF-BPs.

Böni-Schnetzler et al. (1989) demonstrated that insulin therapy of streptozotocin-diabetic rats increased IGF-I mRNA levels in liver and reduced elevated IGF-BP-2 mRNA levels. According to this group, insulin influences the secretion of GH which is a well established stimulator of IGF-I production and a suspected inhibitor of IGF-BP-2.

From other experiments on hypophysectomized and/or fasted versus diabetic rats, Ooi et al. demonstrated a differential induction of IGF-BP-1 and IGF-BP-2 mRNA occurring at different levels of insulin deficiency (Ooi et al., 1990). They showed that BP-2 mRNA increased in mild insulin deficiency such as hypophysectomy and fasting, whereas BP-1 mRNA increased in severe insulin deficiency such as in diabetics. Therefore IGF-BP-1 and IGF-BP-2 considered as the low molecular weight IGF-BPs may have unique biological functions during catabolic states in adult rats (Ooi et al., 1990).

Glucagon, which antagonizes insulin's actions, is present at high levels in diabetic insulin-dependent states (Nair et al., 1987). Since there is a regulatory link between insulin and IGF-I, as demonstrated by Böni-Schnetzler et al. (1990), it is of great interest to study the effects of glucagon on hepatic IGF-BP production. The liver is one of the target organs of

insulin and it is also the major organ responsible for the synthesis and secretion of serum IGF-I. The *in vitro* effects of glucagon on both IGFs and IGF-BPs has not been previously demonstrated. Our system, designed to maintain hepatocytes in culture free of endogenous mitogens, permitted such study.

Glucagon is an important metabolic hormone and together with insulin regulates blood glucose homeostasis during the post-absorptive and the fasted states (Unger et al., 1970). When plasma glucose levels fall, there is a stimulation of glucagon secretion from  $\alpha$ -cells of the pancreas (Gerich et al., 1973). In the insulin-dependent diabetic state, plasma glucose levels stay high and inhibit the secretion of glucagon which in turn affects amino acid metabolism (Nair et al., 1987).

My project has contributed to the IGF-field by describing the hormonal regulation of IGF-BPs secreted from cultured rat hepatocytes. We have shown that the regulation of hepatic IGF-I, IGF-BP-1, and IGF-BP-2 production in cultured rat hepatocytes is under GH and glucagon control.

## **CHAPTER 2: MATERIALS AND METHODS**

### **2.1 Animals**

Sprague Dawley rats weighing 180-200 g were obtained from Charles River Canada Inc. (St. Constant, Quebec, Canada). Rats were housed under standardized lighting (light on from 0700-2000 h) and fed on Purina Chow (Ralston-Purina, St. Louis, MO) prior to study.

### **2.2 Materials and Chemicals**

IGF-I was a generous gift from Ciba-Geigy Ltd. (Basel, Switzerland) and Chiron Corp. (Emeryville, CA). Human prolactin (PRL), corticotropin releasing hormone (CRH) and IGF-II was kindly provided through the Hormone Distribution Program of the National Institutes of Health (Bethesda, MD). Porcine Insulin was purchased from Lilly (Montreal, QUE). Antibodies against rIGF-BP-1 were kindly provided by Dr. L.J. Murphy (Winnipeg, MA) and those against rIGF-BP-2 were kindly provided by Dr. M.M. Rechler (Bethesda, MD). Glucagon, protamine sulphate, activated charcoal (untreated powder) 100-400 mesh, Tris[hydroxymethyl] amino methane (Trizma HCl), bovine serum albumin (BSA), glycine, HEPES (N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]), ornithine, L-lactic acid, selenium, nonidet-P40, EGTA [ethylene glycol-bis ( $\beta$ -aminoethyl ether) N,N,N',N'-tetraacetic acid], Heparin, Tween 20 (polyoxy-ethylenesorbitan monolaurate), Triton X-100 (polyoxyethylene ether), ethanolamine, and anti-rabbit IgG were purchased from Sigma Chemical Co. (St. Louis, MO). Methanol, sodium chloride and hydrochloric acid were purchased from Anachemia (Montreal, QUE). Protein A Sepharose CL-4B, blue dextran dye and Sephadex G-75 (fine) were purchased from Pharmacia Fine Chemicals LKB (Baie d'Urfee, QUE). Bovine GH (USDA-bGH-B-1, 1.4 IU/mg) was kindly supplied by the USDA Animal Hormone Program (Beltsville, MD). Collagenase was purchased from Worthington Biochemical Corporation (Freehold, NJ). Dulbecco's Modified Eagle's Medium (DMEM), Ham's F12 nutrient mixture, and fetal bovine serum (FBS) were from GIBCO (Grand Island,

NY). Whatmann 3MM filter paper was purchased from Fisher (Toronto, ONT). Tissue culture plates (100 mm), Penicillin, Streptomycin, and Fungizone were from Flow Laboratories (Rockville, MD). Collagen (Vitrogen 100) was from Collagen Corporation (Palo Alto, CA). Carrier free  $\text{Na}^{125}\text{I}$  was purchased from New England Nuclear Corp. (Boston, MA). Reagents for polyacrylamide gel electrophoresis [Sodium dodecyl sulfate (SDS), Dithiothreitol (DTT), Low molecular weight standards, Acrylamide 99.9%) were purchased from Bio-Rad (Richmond, CA). The electroblotting unit for transferring protein to nitrocellulose membranes and the electrophoretic unit were purchased from Bio-Rad (Richmond, CA). Nitrocellulose membranes were purchased from Millipore (Bedford, MA). Centricon-10 microconcentrators were purchased from Amicon (Danvers, MA).

### **2.3 Preparation of Primary Hepatocyte Cultures**

Hepatocytes were isolated from Sprague Dawley rats weighing 180-200 g by perfusion *situ* with collagenase as previously described (Barash et al., 1988; Kachra et al., 1991).

Rats were sedated with ether and than injected with 0.075 ml sodium phenobarbital. Five minutes after the animal was ready for surgery, light anesthesia was maintained with ether. The ventral surface of the rat was washed with ethanol before incision. After incision, the liver was exposed and sutures were placed around the following areas with out ligation:

- (a) inferior vena cava, anterior to animals right kidney
- (b) portal vein (1 cm away from branching point)
- (c) portal vein at branching point (natural hole in surrounding tissue)

The peristaltic pump was setup at 2.3 ml / min. with HEPES buffer, pH 7.85 containing 0.6 mM EGTA and 1.5 IU / ml heparin at 37 C. After the pump was started, cannulation of the portal vein was done by securing it with ligated sutures (b) and (c).

Immediately the inferior vena cava (a) posterior to the kidney was cut and buffer was pumped at a rate of 5 ml / min. The chest cavity was opened and the heart's right atrium was nicked. The superior vena cava was cannulated with the return cannula via the right atrium and secured with ligated intramedic tubing. The inferior vena cava anterior to the kidney was ligated immediately and the platform where rat was on was raised above the level of the buffer(s). The flow rate was increased to 30-40 ml / min. and 50 ml of the buffer was allowed to circulate within the designed cannulation system.

The liver was perfused with a 0.075% collagenase solution for approximately 8 minutes by putting the return tubing into the collagenase container. As time progressed, the liver capsule became more leaky and the overall morphology of the liver changed to a "pale" color.

After the perfusion the rat liver was removed and placed into DMEM/F12 medium containing 10% fetal bovine serum (FBS), 10 mM HEPES, 20 mM  $\text{NaHCO}_3$ , 500 IU/ml penicillin, 500  $\mu\text{g/ml}$  streptomycin, and 1.25  $\mu\text{g/ml}$  fungizone. The cells were gently dissociated with a spatula and dispersed in the surrounding medium and the porta hepatis (white gelatinous mass) was discarded. The cell suspension was strained through two layers of sterile cheesecloth in a 50 ml conical tube and diluted to 40 mls with same medium. The cell suspension was centrifuged three times at 500 rpm for 2 min. at 4 C and the supernatant was aspirated at the end of each spin. For the last two washes the cell pellet was resuspended in same medium but containing the antibiotic mix (penicillin, streptomycin and fungizone).

Cell viability determined by Trypan blue (cell suspension diluted 5:1) was 80% at the onset of culture. Cells were counted with the aid of a coulter counter. Cell densities of 4-5 million cells / 100 mm tissue culture dish were plated. Plates were initially coated with collagen (1% solution) for 1 h at 37 C in a humidifier (5%  $\text{CO}_2$ ). Cells were allowed to attach for 24 hours in collagen plated tissue culture dishes with DMEM / F12 medium containing 10% FCS and the antibiotics.

After the overnight attachment period, the media was changed to serum-free conditions supplemented with 0.4 mM ornithine, 2.25  $\mu\text{g/ml}$  L-lactic acid,  $2.5 \times 10^{-8}$  M selenium, and  $1 \times 10^{-8}$  M ethanolamine for another 48 hours ("zero" time point for incubations) before starting the hormonal treatments (Barash et al., 1988).

## **2.4 Hormonal Stimulation and Media Collection**

72 h after plating cells were treated with or without bGH, glucagon, or bGH plus glucagon. The growth hormone and glucagon concentrations were 300 ng/ml and 500 ng/ml, respectively, in all the experiments except if indicated otherwise. Media were collected at 1, 3, 6, 12 and 24 h after addition of hormones and centrifuged at 1000 rpm for 10 min to remove cell debris. The clear supernatants were frozen at  $-20^\circ\text{C}$  prior to further analysis.

## **2.5 Iodination of IGF-I**

IGF-I was labeled with  $\text{Na } ^{125}\text{I}$  by chloramine T procedure to a specific activity of 130  $\mu\text{Ci} / \mu\text{g}$  as previously described (Conover et al., 1989).

## **2.6 Charcoal Binding Assay for IGF-BPs**

IGF-I binding to IGF-BPs in conditioned media was determined by a charcoal binding assay using protamine sulfate to facilitate the  $^{125}\text{I}$ -IGF binding to IGF-BPs (Costigan et al., 1988; Conover et al., 1989; Powell et al., 1989). Labeled  $^{125}\text{I}$ -IGF-I (10,000 cpm) was incubated overnight at  $4^\circ\text{C}$  in a 0.5 ml mix of 50 mM Tris-HCl, 0.5% BSA (pH 7.4) and increasing volumes of conditioned media. After overnight incubation, 1 ml of 1% activated charcoal solution containing 0.2 mg/ml protamine sulfate was added to each tube. All tubes were vortexed and allowed to equilibrate for 10 min. at  $4^\circ\text{C}$  prior to centrifugation. Centrifugation at 3000 rpm at  $4^\circ\text{C}$  for 30 min. was performed and free  $^{125}\text{I}$ -IGF-I in the charcoal pellet was counted. The amount of radiolabeled IGF bound to binding proteins in the supernatant was calculated.

## **2.7 Sephadex G-75 Acid Gel Chromatography**

Dissociation of IGFs from binding proteins in the conditioned media was effected by acid gel filtration (Romanus et al., 1986). A 30 ml column was packed with Sephadex G-75 (allowed to swell for 3 h at 100 C) at 4 C. The column (1.2 x 30 cm; bed volume, 33 ml) was allowed to equilibrate at 4 C prior to calibration with blue dextran and  $^{125}\text{I}$ -IGF-I in 0.01 N HCl, pH 2.2. The equilibrated column was washed with 0.01 N HCl (twice the total bed volume) before sample application. 300  $\mu\text{l}$  of conditioned media were applied to the column and elution was carried out with 0.01 N HCl. 0.5 ml volumes were collected (14 drops/tube) and each fraction was assayed for IGF-BPs by the charcoal binding method and for protein by optical density at 280 nm using a Beckman DU-8 spectrophotometer.

## **2.8 Specificity of IGF-BPs**

The binding proteins resolved from IGFs by Sephadex G-75 acid gel chromatography were assayed for specificity with respect to both IGF-I and IGF-II as well as ACTH, hPRL, glucagon, insulin, and bGH. For this,  $^{125}\text{I}$ -IGF-I (50,000 cpm) was incubated with increasing concentrations of unlabeled peptides in 50 mM Tris-HCl, 0.5% BSA (pH 7.4) at a final volume of 0.5 ml. After overnight incubation 1 ml of 1% activated charcoal solution containing 0.2 mg/ml protamine sulfate was added to each tube. All tubes were vortexed and allowed to equilibrate for 10 min. at 4 C prior to centrifugation. Centrifugation at 3000 rpm for 30 min performed at 4 C separated free  $^{125}\text{I}$ -IGF-I which bound the charcoal pellet and bound  $^{125}\text{I}$ -IGF-I in the supernatant.

To determine the number of the high affinity binding sites present in the media from cultured rat hepatocytes, data from dose-dependent inhibition studies were analyzed using the computerized Ligand software (Munson, 1983), assuming a two binding site model (Baxter and Martin, 1987).

## 2.9 Western Blotting (WB) for IGF-BPs

### 2.9.1 Sample Preparation

All samples were concentrated by the Amicon micro-concentrator method. Centricon-10 concentrators were used (10,000 M.W. cut off). Equal volumes of non-concentrated samples were centrifuged at 5000 rpm for 30 minutes (JA 21 rotor, Beckman J2-21 centrifuge). The number of spins was determined by the degree of concentration required for each experiment. Protein was determined before and after the concentration steps and concentrated media were stored at  $-20^{\circ}\text{C}$  prior to further analyses.

Samples were diluted in sample buffer (table 1):

**Table 1: Sample buffer composition**

|                             | STOCK [ ] | FINAL [ ] | Vol. or<br>Weight / 10 ml |
|-----------------------------|-----------|-----------|---------------------------|
| GLYCEROL                    | 100 %     | 10 %      | 3.0 ml                    |
| SDS IN UPPER<br>TRIS BUFFER | 20 %      | 2.3 %     | 3.45 ml                   |
| UPPER TRIS                  | 0.5 M     | 0.0625 M  | 0.3 ml                    |
| BROMOPHENOL<br>BLUE         | 0.1 %     | 0.001 %   | 0.3 ml                    |
| * DTT                       | SOLID     | 0.1 M     | 0.464 g                   |

Add 2.95 ml of double distilled water to a final volume of 10 ml

\* Addition of DTT produces reducing conditions which was used for the electrophoresis of the low molecular weight standards but not for the IGF-BP's in the conditioned media.

Double distilled water was used throughout the experiments unless otherwise stated. Glassware was washed with regular detergent and rinsed thoroughly with double distilled water before use. Only materials for tissue culture (media, utensils, bottles, etc.) were autoclaved before use.

### 2.9.2 Molecular Weight Markers

There are high and low molecular weight markers available commercially. The use of pre-stained molecular weight markers was not recommended for band sharpness, therefore we used normal (not stained) molecular weight markers in a 1/20 dilution:

5  $\mu$ l marker + 70  $\mu$ l water + 25  $\mu$ l sample buffer (3x conc.)

In addition to low molecular weight markers, control lanes were run with rat plasma and rat serum in order to compare the species of binding proteins present in the circulation vs those present in the media from cultured adult rat hepatocytes. Approximately 2-3  $\mu$ l of serum and/or plasma were diluted in 100  $\mu$ l buffer for SDS-PAGE.

### 2.9.3 SDS-PAGE Electrophoresis

After concentration, samples were subjected to SDS-PAGE using 12.5% acrylamide as described elsewhere (Barenton et al., 1988). Before samples were loaded into the wells, they were boiled for 5 min. at 100<sup>0</sup> C. This is a very important step, and if neglected, the proteins in the sample will aggregate and will not enter the stacking gel. Heat and SDS will denature (unfold) proteins.

Gels made for electrophoresis consisted of a lower (resolving) and upper (stacking) gel phase. The lower phase was loaded first and allowed to polymerize before the upper phase was added.

**Table 2: Resolving gel composition (12.5% Polyacrylamide)**

| <b>COMPOUNDS</b>                   | <b>1 GEL<br/>(ml)</b> | <b>2 GELS<br/>(ml)</b> | <b>3 GELS<br/>(ml)</b> | <b>4 GELS<br/>(ml)</b> |
|------------------------------------|-----------------------|------------------------|------------------------|------------------------|
| <b>Acrylamide<br/>bis (30:0.8)</b> | <b>12.5</b>           | <b>25.0</b>            | <b>37.5</b>            | <b>50.0</b>            |
| <b>Lower tris</b>                  | <b>7.5</b>            | <b>15.0</b>            | <b>22.5</b>            | <b>30.0</b>            |
| <b>Water</b>                       | <b>9.76</b>           | <b>19.56</b>           | <b>29.28</b>           | <b>39.04</b>           |
| <b>TEMED</b>                       | <b>15 µl</b>          | <b>30 µl</b>           | <b>45 µl</b>           | <b>60 µl</b>           |
| <b>10 % APS<br/>Add LAST</b>       | <b>225 µl</b>         | <b>450 µl</b>          | <b>675 µl</b>          | <b>900 µl</b>          |

**TEMED** : N,N,N',N'-tetramethylenediamine (cross-linking agent)

**APS** : Ammonium peroxydisulfate (oxidizing agent)

The gel solution was prepared as shown in table 2 and loaded in the gel cassette at an approximate liquid height of 11.5 cm. On top of this 1-2 ml of a solution (20% methanol + 0.001% bromophenol blue) was added to level the upper surface and to prevent the gel from drying. The gel polymerized in approximately 30-60 min.

Before the stacking gel was loaded, the bromophenol-methanol solution was removed and the upper surface of the polymerized gel was thoroughly rinsed with water.

**Table 3: Stacking gel composition (1% Polyacrylamide)**

| COMPOUNDS                  | 1 GEL<br>(ml) | 2 GELS<br>(ml) | 3 GELS<br>(ml) | 4 GELS<br>(ml) |
|----------------------------|---------------|----------------|----------------|----------------|
| Acrylamide<br>bis (30:0.8) | 1.0           | 2.0            | 3.0            | 4.0            |
| Upper tris                 | 2.5           | 5.0            | 7.5            | 10.0           |
| Water<br>(d.distilled)     | 6.39          | 12.78          | 19.17          | 25.56          |
| TEMED                      | 6.5 $\mu$ l   | 13 $\mu$ l     | 19.5 $\mu$ l   | 26 $\mu$ l     |
| 10 % APS<br>Add LAST       | 100 $\mu$ l   | 200 $\mu$ l    | 300 $\mu$ l    | 400 $\mu$ l    |

TEMED : N,N,N',N'-tetramethylenediamine (cross-linking agent)

APS : Ammonium peroxydisulfate (oxidizing agent)

Ten or 15-well combs were introduced (only half way down), then the stacking gel solution prepared as shown in table 3 was slowly pored in the gel cassette to avoid introduction of air bubbles. Air bubbles must be avoided because they can affect protein migration during electrophoresis and cause band distortion. This was also a concern when removing comb(s) from the prepared gel. Washing of wells with electrophoretic buffer helped remove any additional bubbles formed at the surface of wells.

Usually the stacking gel polymerization period was longer (1 hour) than the resolving gel (30 min to 1 h). After polymerization, gels were ready for sample loading. The electrophoretic buffer was prepared as shown in table 4 and was cooled to 4 C before use. Cold electrophoresis buffer helps reduce the "voltage-dependent heating" of the solution during SDS-PAGE .

**Table 4: Electrophoresis buffer**

|          |        |        |
|----------|--------|--------|
| Tris-HCl | 12.1 g | 0.25 M |
| Glycine  | 57.6 g | 0.19 M |
| SDS      | 4.0 g  | 0.1 %  |

in 4 liters of water

**2.9.4 Electroblotting**

The proteins separated by SDS-PAGE were transferred to nitrocellulose paper prior to incubation with the radiolabeled IGF-I. Transfer buffer was made as shown in table 5 and was cooled to 4 C prior to use.

**Table 5: Transfer buffer (TB)**

|          |        |        |
|----------|--------|--------|
| TRIS     | 12.1 g | 0.25 M |
| GLYCINE  | 57.6 g | 0.19 M |
| METHANOL | 800 ml | 5 %    |

in 4 liters of H<sub>2</sub>O

The gel was soaked for 20 min. in transfer buffer and allowed to equilibrate. Washing the excess SDS off the gel (container # 1) improved protein transfer. Then a 5'x7' piece of nitrocellulose transfer paper was cut and immersed first in methanol and then in water. Two rectangular sponges were soaked in transfer buffer (container #2). The electroblotting rack was placed in a third container with the GREY colored side in the transfer buffer.

The "sandwiching" occurred in the following order from bottom to top:

- 1) sponge
- 2) 3MM filter paper
- 3) SDS-PAGE gel

- 4) nitrocellulose sheet
- 5) 3MM filter paper
- 6) sponge

It was assured that no bubbles were present since transfer of proteins did not occur under the area of a bubble. It was also made sure that the grey side of the rack faced the grey sticker on the outside of the electroblotting apparatus, or else the proteins did not transfer from the gel into the nitrocellulose but rather transferred into the transfer buffer (TB).

The electrotransfer was performed overnight with the BIO-RAD apparatus at 0.18 mA at 4<sup>0</sup> C and at 0.3 mA for further 3 hours next morning at 4<sup>0</sup> C .

After transfer, the nitrocellulose paper was stained in 0.2% red Ponceau dye for about 10 min. This procedure stains proteins on nitrocellulose without denaturing them and serves as a control for the degree of transfer.

Before "blocking" the nitrocellulose with solutions described below, the molecular weight markers (stained by the dye) were cut and dried. A buffered saline solution (BSS) was prepared (0.15 M NaCl, 10 mM Tris-HCl, pH 7.40) before incubations with solutions described below.

The nitrocellulose membrane was prepared for ligand blotting and was incubated with the following series of solutions:

- a) balanced salt solution (BSS) + 3% nonidet P-40 (NP-40); 30 min., 4<sup>0</sup> C on shaker
- b) balanced salt solution (BSS) + 3% BSA ; 2 hours, 4<sup>0</sup> C on shaker
- c) balanced salt solution (BSS) + 0.1% tween-20; 10 min., 4<sup>0</sup> C on shaker

Then, the nitrocellulose membrane was incubated overnight at 4<sup>0</sup> C with 400,000 cpm's <sup>125</sup> I-IGF-I / 40 ml of (BSS + 1% BSA & 0.1% Tw-20). After the incubation period, the nitrocellulose was washed with the following solutions:

- 1) balanced salt solution (BSS) + 0.1% Tween-20 (2x 15 min.)
- 2) balanced salt solution (BSS) (3x 15 min.)

Then nitrocellulose membrane was allowed to dry at room temperature for 1 hour and was then exposed to Kodak X-ray film at  $-70^{\circ}\text{C}$  for approximately 4-5 days (Costigan et al., 1988; Hossenlopp et al., 1986).

## **2.10 Immunoprecipitation of IGF-BPs Followed by Western Botting**

Aliquot(s) of 250  $\mu\text{l}$  of concentrated media were incubated overnight at 4 C on a shaker with 5-10  $\mu\text{l}$  of antibody against either rIGF-BP-1 or rIGF-BP-2. Each tube received 75  $\mu\text{l}$  of protein-A Sepharose and was incubated for 2 h at 4 C on shaker. Centrifugation of samples in a microfuge centrifuge (1000 rpm, 1 min.) removed most of the immunoprecipitated material.

The pelleted protein-antibody-(protein-A) Sepharose complex was washed and centrifuged twice with solution A (see below) and once with solution B (see following page) before preparation for SDS-PAGE followed by Western blotting (Yang et al., 1990).

### **SOLUTION A**

|                     |        |        |
|---------------------|--------|--------|
| 10% TritonX-100     | 5.0 ml | 1.0%   |
| 10% SDS             | 0.5 ml | 0.1%   |
| 50 mM HEPES, pH 7.4 | 5.0 ml | 5.0 mM |

( + 38.5 ml  $\text{H}_2\text{O}$  in  $V_t = 50\text{ ml}$  )

**SOLUTION B**

|                     |         |        |
|---------------------|---------|--------|
| 10% TritonX-100     | 1.0 ml  | 0.1%   |
| 50 mM HEPES, pH 7.4 | 10.0 ml | 5.0 mM |

( + 89 ml H<sub>2</sub>O in V<sub>t</sub> = 100 ml )

**2.11 Densitometry**

Autoradiograms were analyzed by densitometry using an Ultrascan XL laser densitometer, and absorbance curves were integrated and compared. The  $M_r$  was determined using Gelscan XL software (Pharmacia LKB Biotechnology, QUE).

## CHAPTER 3: RESULTS

### 3.1 IGF-BP secretion in conditioned media by adult rat hepatocytes cultures

Hepatocytes are known to secrete insulin-like growth factors (IGFs) and their binding proteins into the culture medium (Scott et al., 1985a; Scott et al., 1985b; Scott et al., 1986). In our primary hepatocyte cultures, we determined that IGF-BPs are secreted into the medium using the charcoal binding assay method as described in Materials and Methods.  $^{125}\text{I}$ -IGF-I binding increased in proportion to the volume of medium assayed (Fig. 8). A higher  $^{125}\text{I}$ -IGF-I binding was observed in the presence of protamine sulfate. The data was in accordance with other reports (Conover et al., 1989; Powell et al., 1987).

Control (non-treated) hepatocytes produced IGF-BPs. This was unchanged by adding glucagon. However, bovine growth hormone (bGH) inhibited IGF-BP secretion (Fig.9). In the presence of glucagon, the inhibitory effect of bGH was overcome. The data of three separate experiments are summarized in table 6. Note that here we observe a stimulatory effect of glucagon compared to control.

In order to identify the IGF-binding proteins secreted by adult rat hepatocytes, the conditioned media were concentrated before being subjected to SDS-PAGE and Western ligand blotting. A 10-fold concentration by lyophilization was performed on conditioned media from the cultured cells and the concentrated material was resuspended in either 0.01 N HCl or PBS as seen in Figs. 10 & 11. The inhibitory effect of GH was less pronounced when the samples were lyophilized. Therefore concentration by lyophilization was not employed hereafter.

A second method used to concentrate samples was by Centricon-10 or Centriprep-10 micro and macroconcentrators. Assays on such concentrated samples were closely comparable to the results in non-concentrated samples. Therefore, Centricon-10 was used to concentrate the

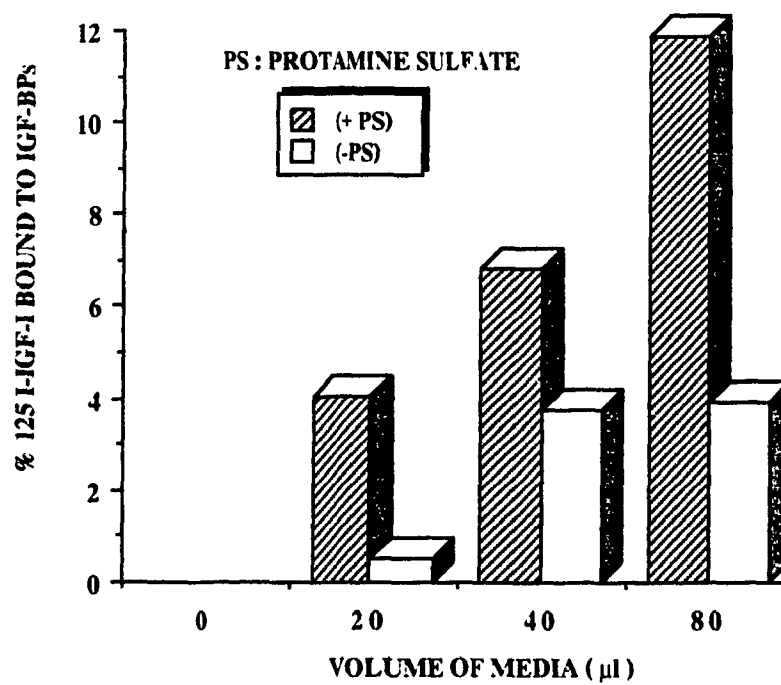


Fig. (8). The IGF-I binding activity secreted by adult hepatocytes in culture.

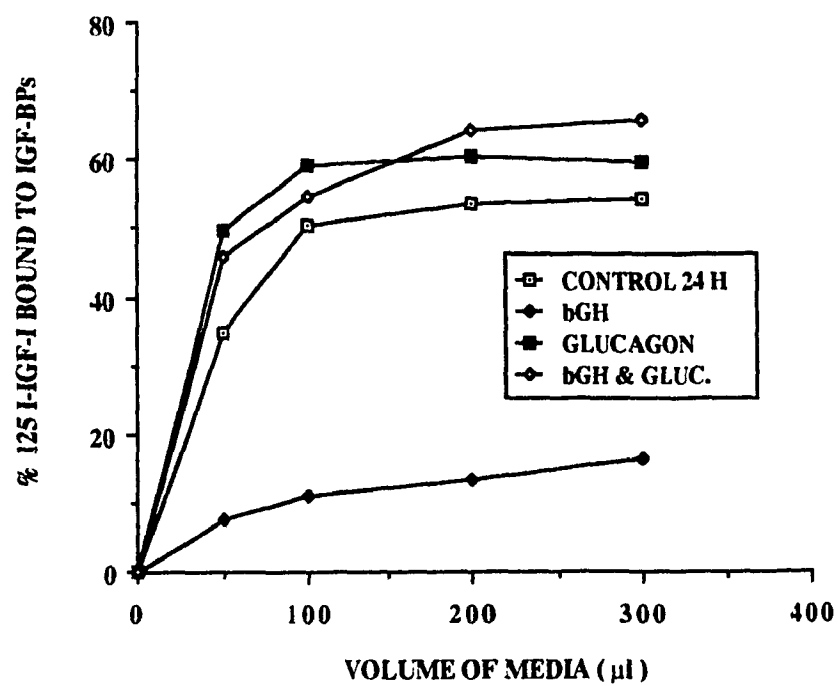


Fig. (9). Effect of bGH and/or glucagon on secretion of IGF-BPs by cultured rat hepatocytes after 24 hours of incubation

Table (6). The effect of bGH and/or glucagon on IGF-BP accumulation in media from cultured rat hepatocytes. Each value is the mean  $\pm$  SD of three separate incubations.

| TREATMENT      | IGF-BP<br>(% [ $^{125}$ I]IGF-I BOUND) |
|----------------|--|
| CONTROL        | $26.76 \pm 3.92$                       |
| bGH            | $3.20 \pm 2.30^a$                      |
| GLUCAGON       | $40.64 \pm 4.46^b$                     |
| bGH + GLUCAGON | $37.70 \pm 3.04^c$                     |

<sup>a</sup>  $P \leq 0.0005$  compared to control.

<sup>b</sup>  $P \leq 0.01$  compared to control.

<sup>c</sup>  $P \leq 0.01$  compared to control.

40  $\mu$ l aliquots.

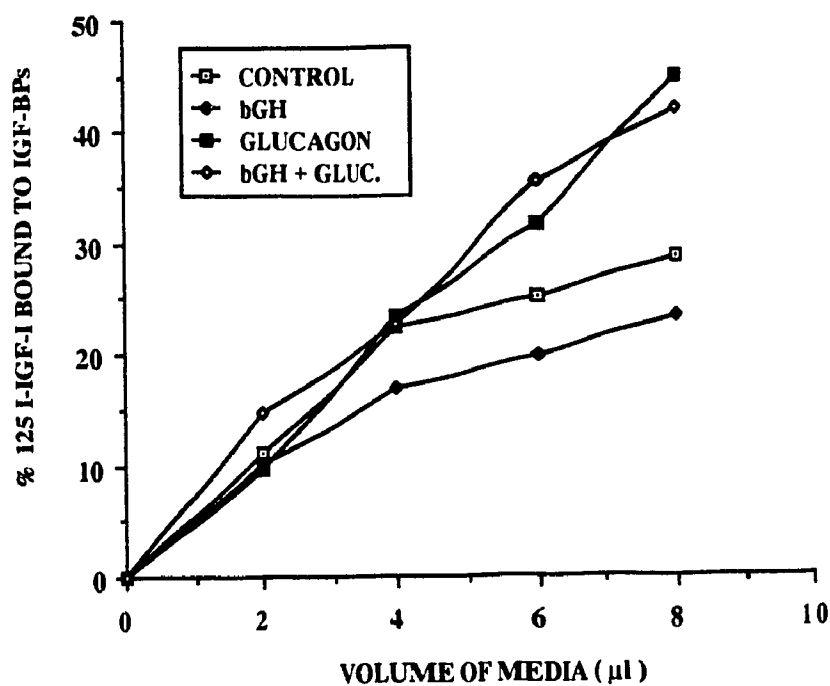


Fig. 10. Effect of lyophilization of media (10x 0.01 N HCl) on IGF binding to IGF-BPs secreted by the rat hepatocytes

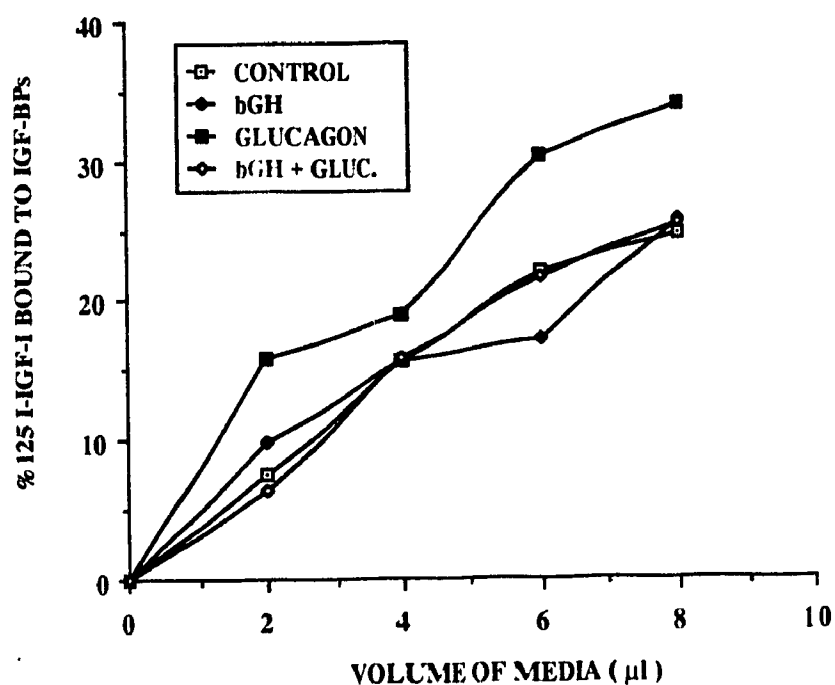


Fig. 11. Effect of lyophilization of media (10x 0.01 N PBS) on IGF binding to IGF-BPs secreted by the rat hepatocytes in culture

samples. This technique for concentration was employed in all the subsequent studies reported in this thesis.

We have not explored the reason why lyophilization reduces the apparent result with GH. However we suggest that part of the reason may be that the IGF-BPs are partially denatured by the lyophilization procedure. Therefore the higher control levels of IGF-BP are more markedly decreased than the already low levels in the media from GH-treated hepatocytes.

### **3.2 Western blotting**

The identification of the different species of IGF-binding proteins secreted by adult rat hepatocyte cultures was assessed by Western blotting. Fig. 12 shows that the major species of IGF-binding proteins that are secreted by the cells in culture are the 24 kDa and 30-34 kDa complexes. The larger molecular weight complex migrates as a broad band. It is likely that this band is comprised of more than one species of binding protein (Hossenlopp et al., 1987).

In Fig. 12 the effect of GH in inhibiting IGF-BP production by the hepatocytes in culture is striking. The regulation of IGF-BP secretion by bGH, glucagon and the combination of both (shown by ligand blotting in Fig.13) was analyzed densitometrically to quantitate the degree of inhibition of BP secretion into the medium. Again bGH inhibited strikingly the secretion of both 24 kDa and 30-34 kDa IGF-BP species. However, glucagon stimulated BP secretion into the medium by 2-fold and 4-fold for the smaller and larger complexes respectively when compared to control. The inhibitory effect of bGH was reversed in the presence of glucagon (Fig. 13 and Fig.14).

The effect of glucagon and bGH on the secretion of IGF-BPs ( figs. 12 and 13) was comparable to the results from the charcoal binding assay in figure 9. Although in the Western blotts (figs. 12 and 13), GH treatment reveals no IGF-BP signal, in the binding assay (fig. 9)

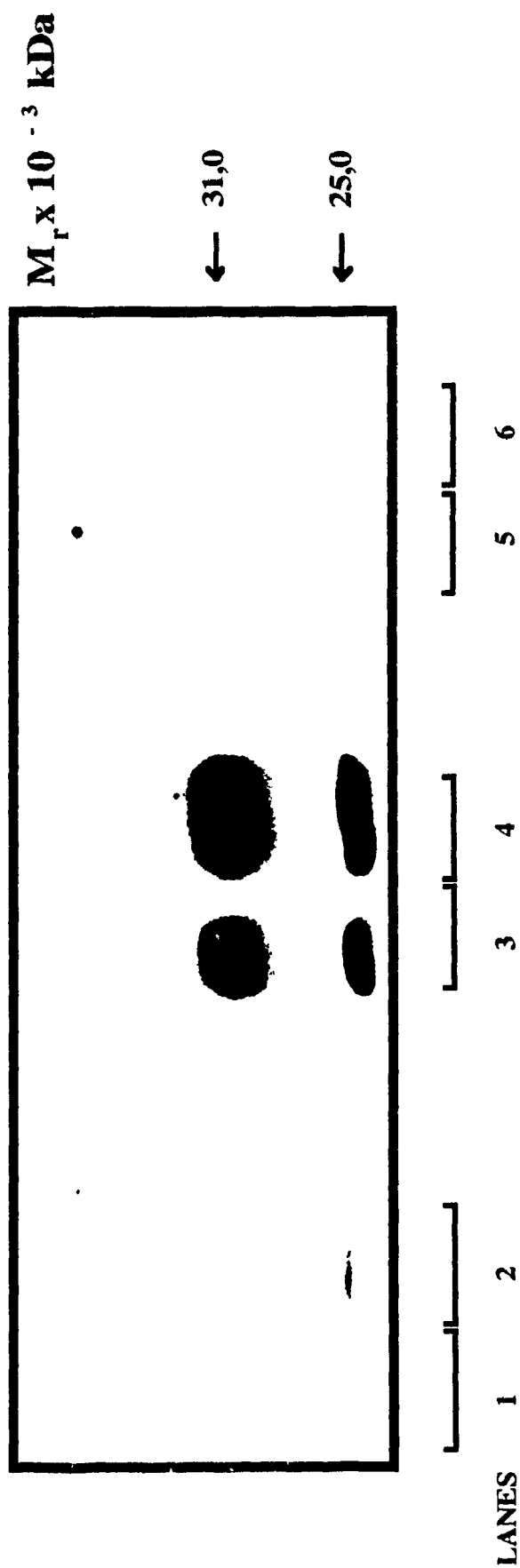


Fig.12 Western blotting of the different species of IGF-BPs secreted by cultured hepatocytes after 24 h of incubation. Lanes 1 and 2 are 3  $\mu$ l of rat serum and plasma respectively. Lanes 3 and 4 are 20 and 40  $\mu$ g of total protein from media of control cultured hepatocytes. Lanes 5 and 6 are 20 and 40  $\mu$ g total protein from media of bGH treated cultured hepatocytes.

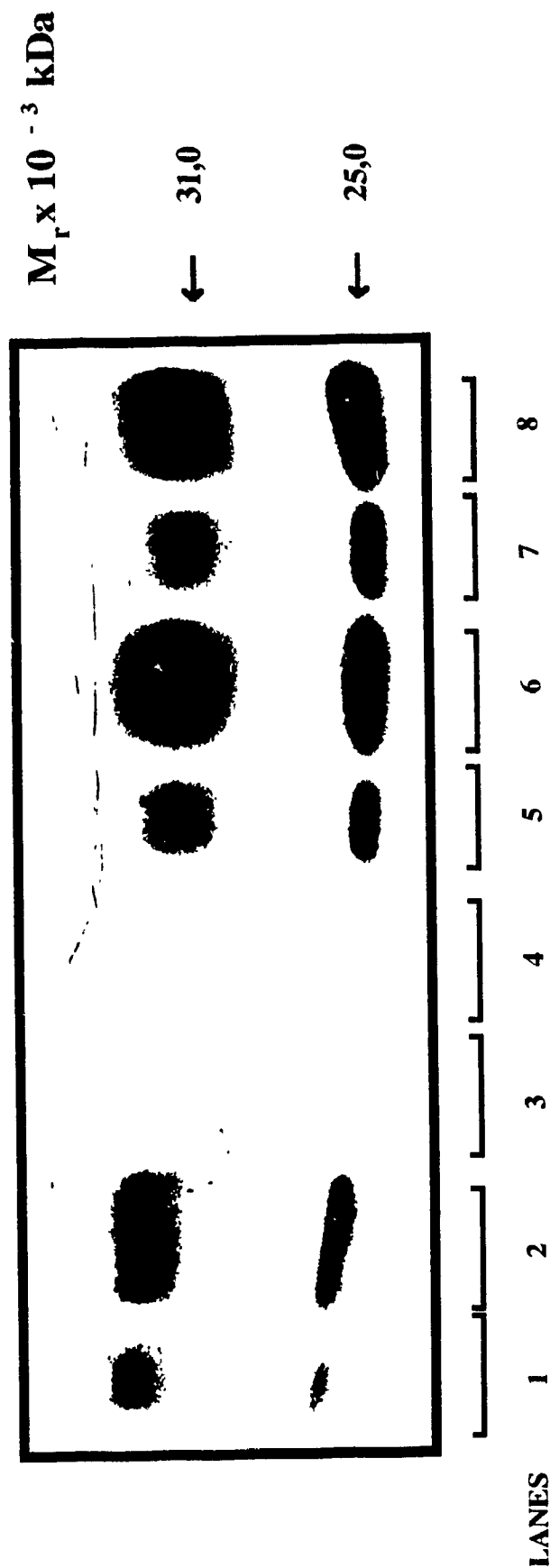


Fig. 13 Effects of bGH and/or glucagon on different species of IGF-BPs secreted by cultured hepatocytes after 24 h of incubation. All lanes consist of 20 and 40  $\mu$ g of total protein respectively. Lanes 1 and 2 are from media of control cultured hepatocytes. Lanes 3 and 4 are from media of bGH-treated cultured hepatocytes. Lanes 5 and 6 are from glucagon treated cultured hepatocytes, and lanes 7 and 8 are from bGH and glucagon treated cells.

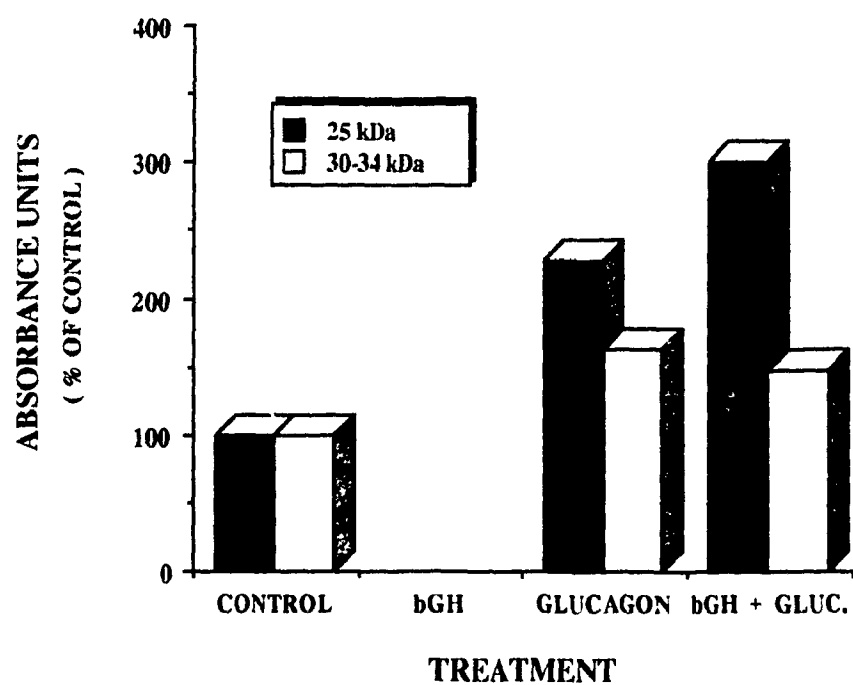


Fig.(14). Densitometric scan of the autoradiograph shown in fig. 13  
Absorbance units are expressed as a percentage of the control value

there is evidence for presence of low levels of IGF-BPs. IGF binding assays are more sensitive than Western blots in detecting protein presence at low levels.

Western ligand blotting analysis of IGF-BPs with  $^{125}\text{I}$ -IGF-I was not possible with samples concentrated (10-fold) by lyophilization. No autoradiographic signal was detected for 24 kDa and 30-34 kDa complexes with either control or hormonally treated hepatocytes.

### **3.3 Time-course of bGH and/or glucagon effect on IGF-BPs secreted by adult rat hepatocytes in primary culture**

IGF-binding protein secretion into the medium increased as a function of time. Fig. 15 shows that the concentration of IGF-BPs increased in conditioned media with time except for growth hormone treated cells.

There were some variations within the different time intervals in assessing quantitatively the hormonal effects. From intervals 1, 3, and 6 hours, the suppressive effect of bGH and stimulatory effect of glucagon on IGF-BP secretion into the media were not observed; whereas by 12 and 24 hours the effects were evident (table 7). Also IGF-BP levels decreased very sharply in bGH treated cells between 12 and 24 hours in serum-free conditions. This may suggest an increase in metabolism of bGH by the hepatocytes during this time period.

Comparable results were obtained in three separate experiments using various volumes of concentrated media as shown in Table 7.

Table 7. Data on bGH and / or glucagon's effect on binding protein secretion by the hepatocytes in culture. Each value represents the mean of three different experiments.

| INCUBATION TIME | CONTROL (% BOUND) | bGH (% BOUND)   | GLUCAGON (% BOUND) | bGH + GLUCAGON (% BOUND) |
|-----------------|-------------------|-----------------|--------------------|--------------------------|
| 1 HOUR          | 1.87 $\pm$ 0.67   | 0               | 3.47 $\pm$ 2.67    | 0.92 $\pm$ 0.11          |
| 3 HOURS         | 5.29 $\pm$ 2.03   | 0               | 6.19 $\pm$ 0.65    | 6.39 $\pm$ 4.75          |
| 6 HOURS         | 5.08 $\pm$ 0.56   | 9.14 $\pm$ 2.67 | 7.45 $\pm$ 2.39    | 5.51 $\pm$ 1.27          |
| 12 HOURS        | 12.73 $\pm$ 3.68  | 9.15 $\pm$ 2.75 | 17.47 $\pm$ 4.76   | 19.43 $\pm$ 1.40         |
| 24 HOURS        | 18.04 $\pm$ 4.97  | 1.33 $\pm$ 0.33 | 29.76 $\pm$ 4.02   | 21.18 $\pm$ 4.80         |

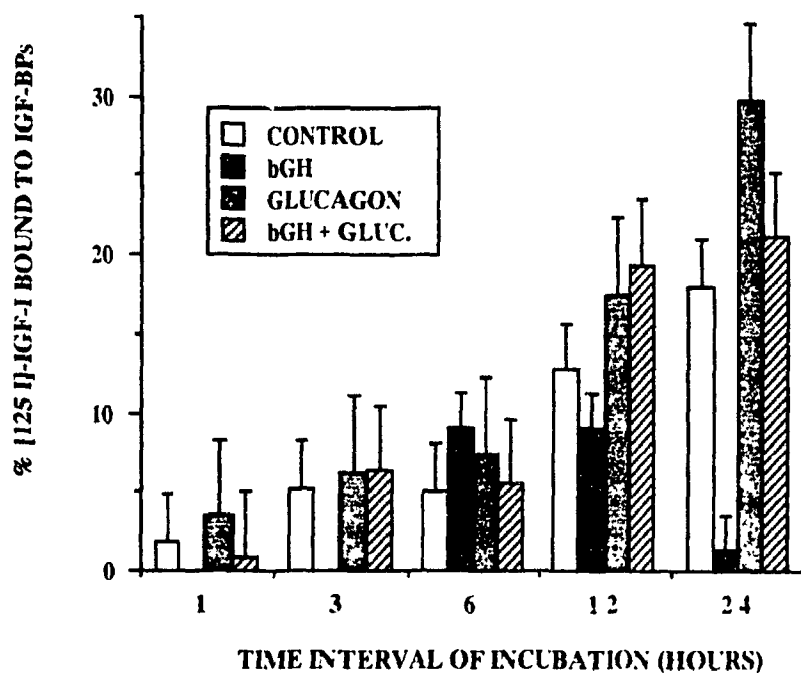


Fig. (15). Time-dependent effect of bGH and/or glucagon on IGF-BPs secreted by cultured rat hepatocytes. Each value is the mean  $\pm$  SD of three separate incubations

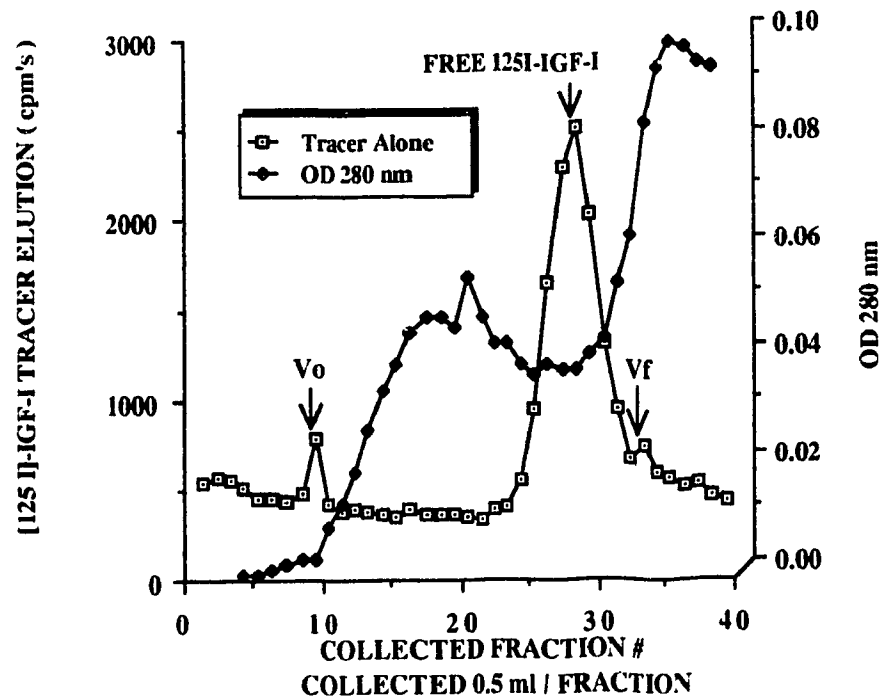


Fig. (16). Analysis of IGF-BPs secreted by the cultured rat hepatocytes by Sephadex G-75 acid gel chromatography. IGF-I tracer (50,000 cpm's) was loaded first in the column with 1% bovine serum albumin. 0.5 ml of control media (24 hour incubation) was applied as described in Materials and Methods. Eluate was collected in 0.5 ml fractions. Protein content was monitored in each fraction by recording the absorbance at 280 nm. Each point represents the mean value of two experiments.

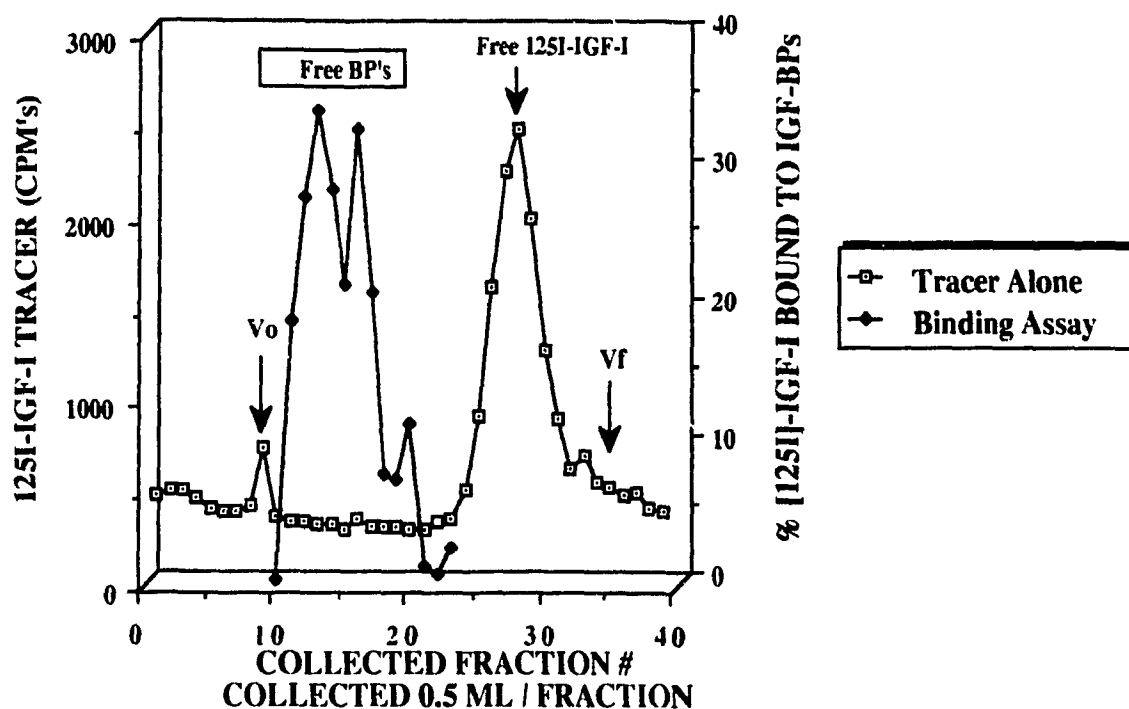


Fig. (17). Analysis of IGF-BPs secreted by the cultured rat hepatocytes by Sephadex G-75 acid gel chromatography. IGF-I tracer (50,000 cpm's) was eluted first through the column with 1% bovine serum albumin. Control media (24 hour incubation) was applied ( $\approx 0.5$  ml) as described in Materials and Methods. Eluate was collected in 0.5 ml fractions. Fractions eluted between the void volume ( $V_o$ ) and the free IGF-I tracer region were assayed for  $^{125}\text{I}$ -IGF-I binding activity using the charcoal assay as described in Materials and Methods. Each point represents the mean value of two experiments.

### **3.4 IGF-BP(s) analysis by gel filtration method**

To investigate the specific binding properties of IGF-BPs secreted by the hepatocytes in culture, the binding proteins were resolved from endogenous IGF(s) by Sephadex G-75 chromatography under acid conditions (0.01 N HCl, pH 2.20) as described in Materials and Methods. Free  $^{125}\text{I}$ -IGF-I eluted between fractions # 25-30 as shown in Fig.16. Spectrophotometric analysis of collected fractions at 280 nm revealed a peak between fractions # 15-20, where elution of proteins between 70 and 10 Kd occurs (Hossenlopp et al., 1987). To verify the presence IGF-BPs within fractions # 11-24 (region where binding proteins do not co-elute with endogenous IGFs), an aliquot of each fraction collected was tested by  $^{125}\text{I}$ -IGF-I charcoal binding assay. Fig. 17 shows the profile of  $^{125}\text{I}$ -IGF-I specific binding to free IGF-BP(s). Two major and a minor  $^{125}\text{I}$ -IGF-I binding activity peaks appeared between fractions # 11-24.

### **3.5 Competitive binding studies of the different species of IGF-BP(s)**

First we assessed whether the two major  $^{125}\text{I}$ -IGF-I binding activity peaks revealed in figure 17 have equal affinity for both tracers. Charcoal binding assays were performed on acid Sephadex G-75 fractionated IGF-BP(s). IGF-I inhibited equipotently  $^{125}\text{I}$ -IGF-I binding to either major peak (Fig. 18). Therefore the two major peaks were pooled and assayed in greater detail for the competitive efficacy of IGF-I (Fig. 19). Competitive inhibition by IGF-I and IGF-II of the binding of  $^{125}\text{I}$ -labelled IGF-I to IGF-BP species was performed. Fig. 19 shows a dose-dependent inhibition of  $^{125}\text{I}$ -IGF-I by unlabeled IGF-I (50% inhibition at  $\approx 1000$  pg/ml and complete inhibition by  $\approx 5000$ -10,000 pg/ml). Fig. 20 shows that both IGF(s) were equipotent in the inhibition of  $^{125}\text{I}$ -IGF-I binding. Maximal displacement of IGF-I tracer was observed with a concentration of 5000 pg/ml cold IGF-I and 10,000 pg/ml of cold IGF-II. Structurally unrelated peptides (GH, prolactin, glucagon, insulin, and ACTH) had no inhibitory effect.

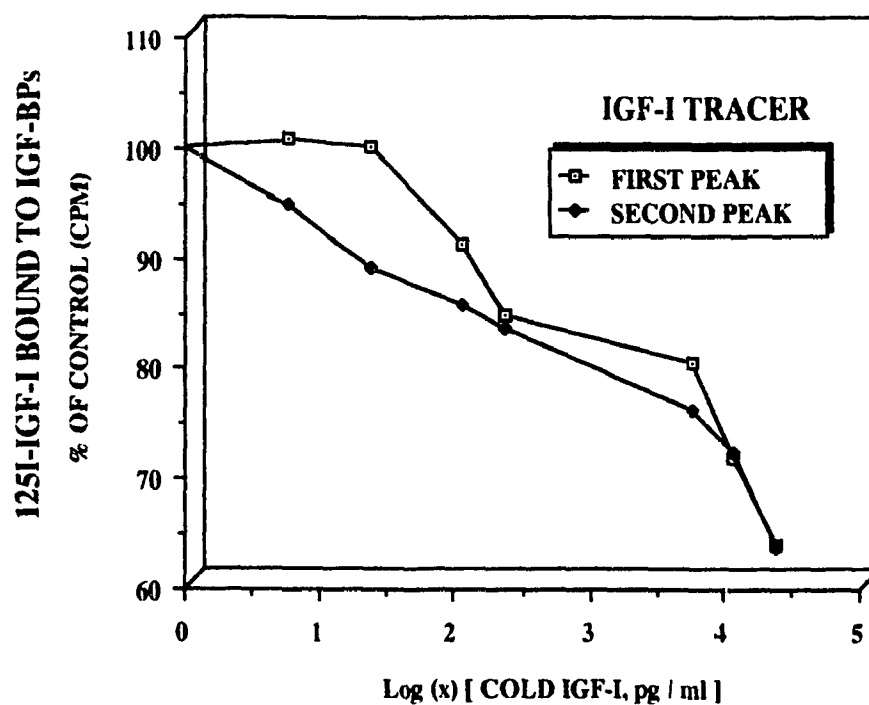


Fig. (18). Dose-dependent inhibition of  $^{125}\text{I}$ -IGF-I binding by different concentrations of unlabeled IGF-I to binding proteins isolated by Sephadex G-75 acid chromatography on media obtained from adult rat hepatocytes. Both  $^{125}\text{I}$ -IGF-I binding activity peaks seen in fig. 17 were assayed separately for tracer specificity as described in Materials and Methods.

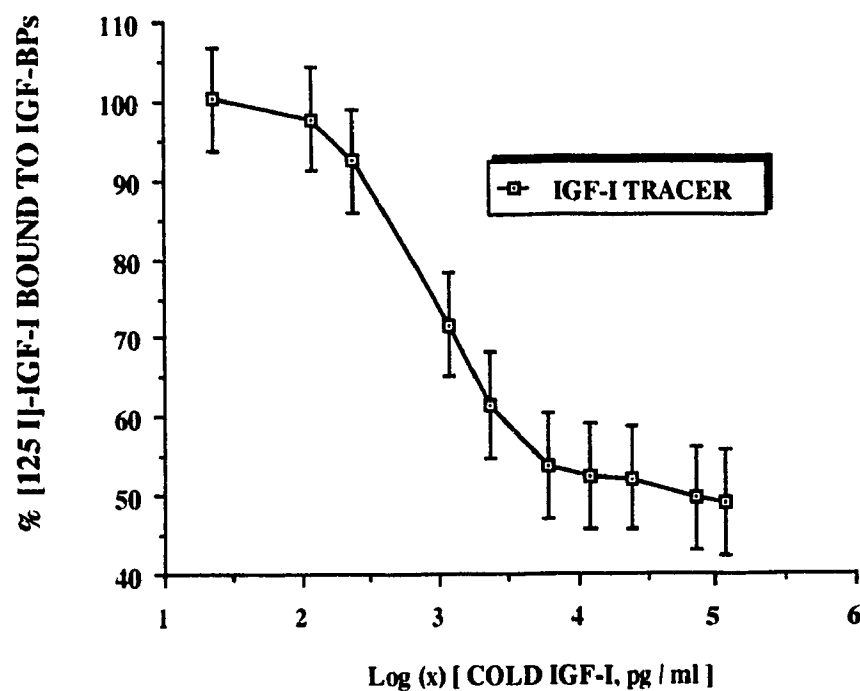


Fig. (19). Dose-dependent inhibition of  $^{125}\text{I}$ -IGF-I binding by different concentrations of unlabeled IGF-I to binding proteins isolated by Sephadex G-75 acid chromatography on media obtained from adult rat hepatocytes. Fractions # 10-24 as seen in fig. 17 were pooled, neutralized, and assayed for tracer specificity as described in Materials and Methods. Each point represents the mean  $\pm$  SD from three assays.

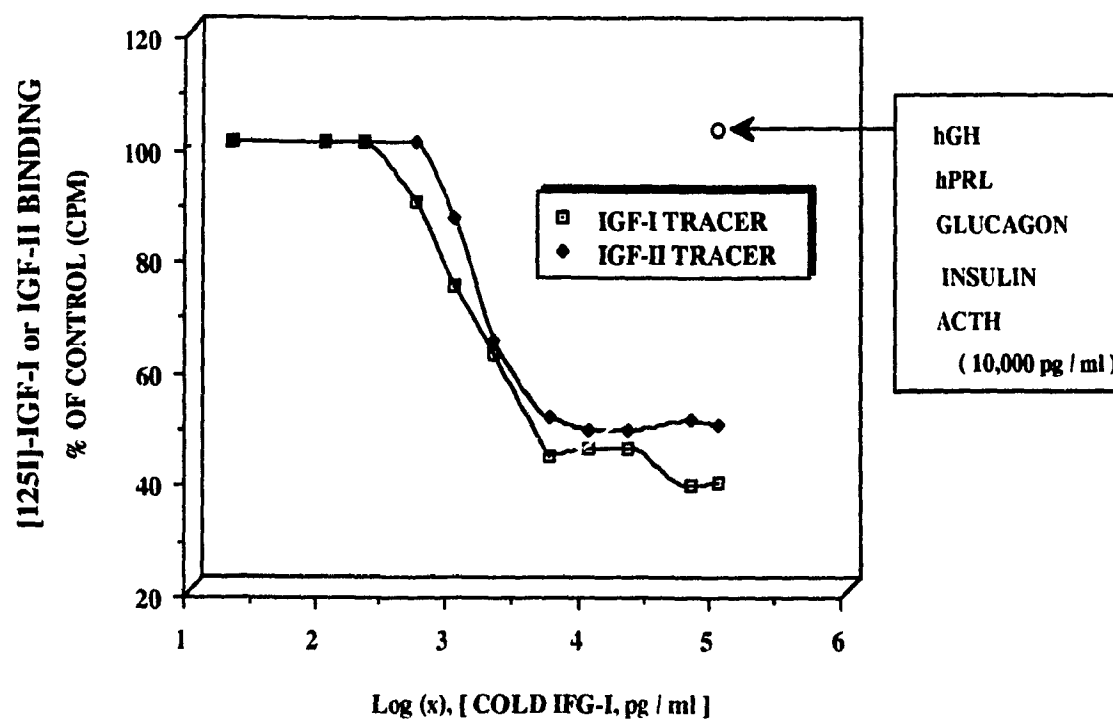


Fig. (20). Dose-dependent inhibition of  $[^{125}\text{I}]$ -IGF-I and  $[^{125}\text{I}]$ -IGF-II binding to acid-stripped binding proteins by different concentrations of unlabeled peptides.

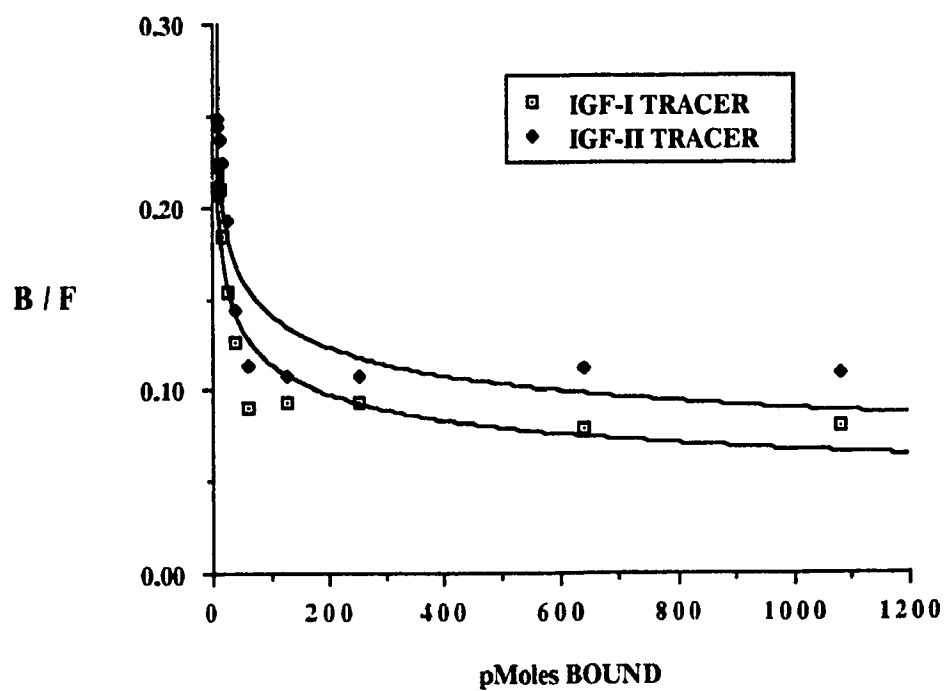


Fig. (21). Scatchard analysis of dose-dependent inhibition data obtained from IGF-BPs secreted by cultured adult rat hepatocytes.

Table 8. Scatchard analysis of IGF-BPs from media of adult rat hepatocytes.

AFFINITY BINDING CONSTANTS ARE DETERMINED WITH EBDA/LIGAND SOFTWARE PROGRAM ON IBM-PC COMPUTER AS DESCRIBED IN MATERIALS AND METHODS.

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**FOR [ $^{125}$ I]-IGF-I AS TRACER  
DISPLACEMENT WITH COLD (IGF-I)**

---

AFFINITY BINDING CONSTANTS :  
(the reciprocal of the dissociation constants)

$$K_1 \text{ [HIGH AFFINITY]} = 1.29 \times 10^{10} \text{ l / mol}$$

$$K_2 \text{ [LOW AFFINITY]} = 2.28 \times 10^5 \text{ l / mol}$$

---

**FOR [ $^{125}$ I]-IGF-I AS TRACER  
DISPLACEMENT WITH COLD (IGF-II)**

---

AFFINITY BINDING CONSTANTS :  
(the reciprocal of the dissociation constants)

$$K_1 \text{ [HIGH AFFINITY]} = 1.34 \times 10^{10} \text{ l / mol}$$

$$K_2 \text{ [LOW AFFINITY]} = 2.25 \times 10^7 \text{ l / mol}$$

Scatchard analyses (see Fig.21) of binding data from IGF-BP(s) indicated a high affinity binding sites ( $K_1[\text{IGF-I}] = 1.29 \times 10^{10} \text{ l/mol}$ ) and ( $K_1[\text{IGF-II}] = 1.34 \times 10^{10} \text{ l/mol}$ ) plus low affinity binding sites ( $K_2[\text{IGF-I}] = 2.28 \times 10^5 \text{ l/mol}$ ) and ( $K_2[\text{IGF-II}] = 2.25 \times 10^7 \text{ l/mol}$ ) as previously reported by Baxter et al. in 1987 (table 8). The high affinity binding sites are not a good estimate of the true number of binding sites due to the presence of multiple binding proteins in our media.

The similarity of the affinities of these IGF-BP(s) complexes for both IGFs has been reported previously (Scott et al., 1985a)

### **3.6 Immunoprecipitation of IGF-BPs prior to western blotting**

Immunoprecipitation of IGF-BPs with rabbit anti-rat IGF-BP-1 or rabbit anti-rat IGF-BP-2 prior to Western blotting resulted in two distinct bands for the 30-30 kDa binding protein complex.

Fig. 22 shows that there is a concentration dependent immunoprecipitation of both binding proteins. Immunoprecipitation with individual antisera resolved the 30-34 kDa broad band seen in previous Western blots into a 31-32 kDa BP-1 and a 35-36 kDa BP-2 bands (see Fig.22: lanes 2-7 and 8-13). Laser densitometry was performed on autoradiograph (Fig. 22) for molecular weight determination and quantitation of signal for both BP-1 and BP-2.

There seems to be some cross-reactivity of the two antibodies for both species of binding proteins. After the control value for non-specific immunoprecipitation by IgG was subtracted from the remaining lanes to determine specific binding (table 9), anti-BP-2 antibody seemed to be more immunoreactive than anti-BP-1 in the media from these adult rat hepatocytes (Fig. 23).

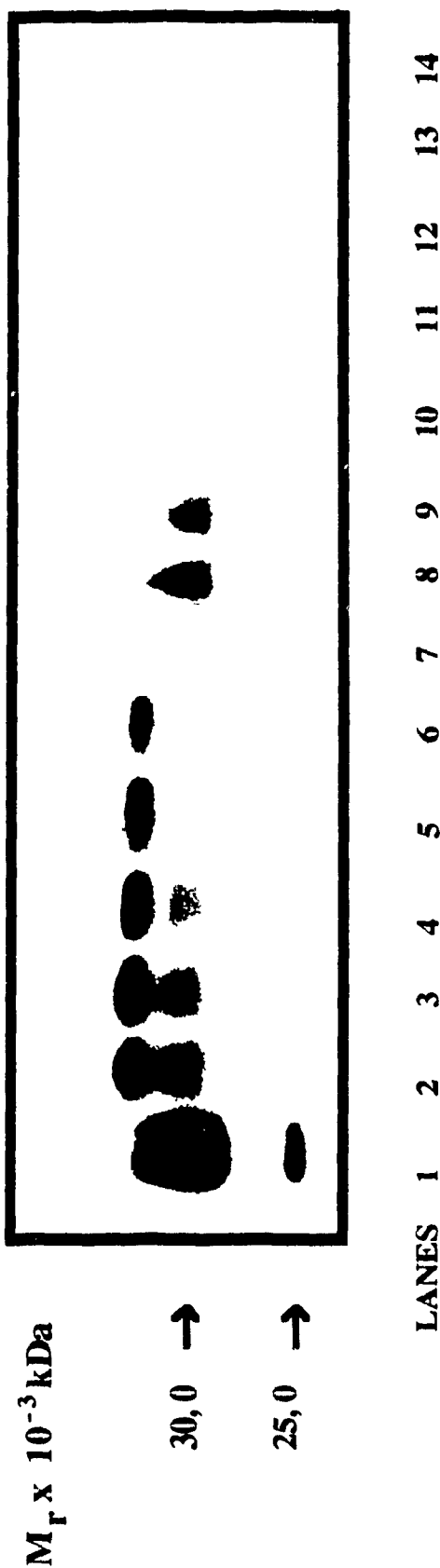


Fig. 22. Immunoprecipitation of media from adult rat hepatocytes using antiserum to rIGF-BP-1 and rIGF-BP-2 prior to ligand blotting. All samples from control media were immunoprecipitated prior to ligand blotting with IGF-I tracer except the first lane. Lane (1) is control [24 hour] media. Lanes (2-7) consists of control media immunoprecipitated with polyclonal antibody against rIGF-BP-2 at the following dilutions ; 1:20, 1:50, 1:100, 1:500, 1:1000, and 1:5000 respectively. The pellet was prepared for SDS-PAGE followed by ligand blot as described in Materials and Methods. Lanes (8-13) consist of control media immunoprecipitated with polyclonal antibody against rIGF-BP-1 with same dilutions as in lanes 2-7. Lane (14) is a control for non-specific binding with rIg-G.

Table 9. Densitometric data from autoradiogram shown in fig.22. Each lane was scanned individually and non-specific Ig G binding was subtracted.

| ANTIBODY DILUTION | IGF-BP-1 ANTIBODY<br>(ABSORBANCE)<br>(AU/mm) | IGF-BP-2 ANTIBODY<br>(ABSORBANCE)<br>(AU/mm) |
|-------------------|--|--|
| CONTROL           | 7.49   | 7.49   |
| 1:10              | 2.09   | 3.17   |
| 1:50              | 0.89   | 2.91   |
| 1:100             | 0.21   | 2.26   |
| 1:500             | --   | 1.69   |
| 1:1000            | --   | 1.15   |
| 1:5000            | --   | 0.26   |

- Non-specific binding (see lane 14 in fig. 22) is already subtracted from all lanes
- Non-specific binding is determined with IgG as control antibody
- The non-specific values obtained from computer assisted densitometry are the following:

1). 30-34 kD complex : 0.64 (AU/mm)

2). 35-36 kD complex : ----

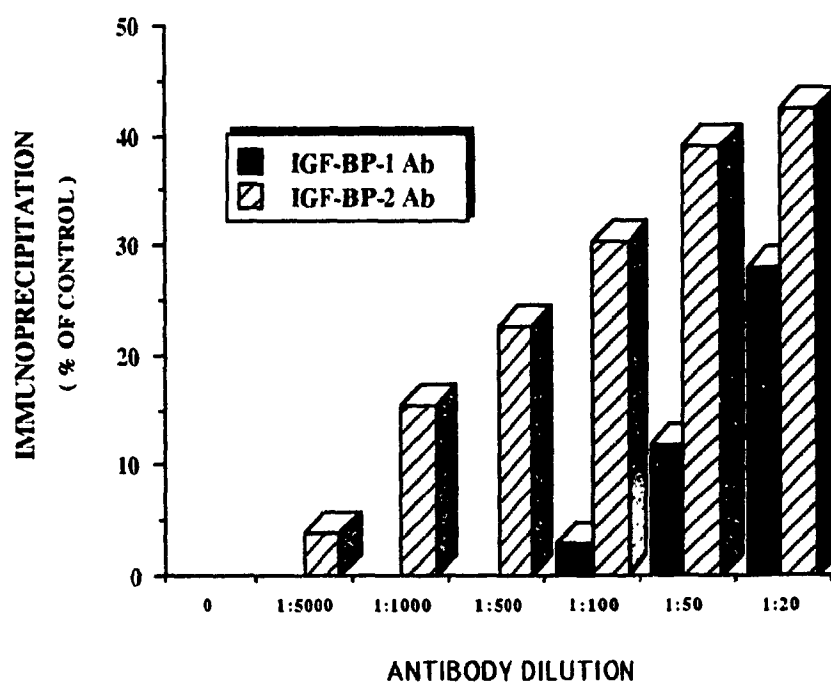


Fig. 23. Immunoprecipitation of IGF-BPs present in the media from adult rat hepatocytes using polyclonal antibodies against rat IGF-BP-1 and IGF-BP-2. The autoradiogram from fig. 22 was scanned densitometrically. Absorbance values were expressed as % of control and used to assess the antibody efficiency for immunoprecipitation.

## **CHAPTER 4: DISCUSSION AND CONCLUSIONS**

Liver produces several binding proteins that have differential affinities for IGFs. IGF-BP-1, IGF-BP-2 and IGF-BP-4 have been considered to be possible local modulators of IGF actions; whereas IGF-BP-3 is composed of a large molecular weight complex found in the circulation. Our adult rat hepatocytes in primary culture secrete insulin-like growth factor (IGF)-binding proteins (IGF-BPs) into their conditioned medium. IGF-BP-1 and IGF-BP-2 are secreted by our hepatocyte cultures as demonstrated in our immunological studies. Although Western ligand blotting showed the presence of a lower molecular weight binding protein, classification of this IGF-BP was not possible from our studies. The striking observation was GH's inhibitory effect on IGF-BP secretion. Glucagon had a stimulatory effect on IGF-BP secretion and when combined with GH the inhibitory effect of GH was reversed.

### **4.1 IGF-Binding Activity**

IGF-binding activity in conditioned media from adult rat hepatocyte cultures was assessed by charcoal adsorption assays in the presence of protamine sulfate. Initially, protamine sulfate (PS) had been used in IGF binding assays because it was able to control the charcoal adsorption rate (Scott et al., 1985). This highly basic molecule when added to the activated charcoal solution, it alters the charge and adsorptive properties of activated charcoal. It is believed to "coat" the charcoal particles and therefore change the overall surface charge. This charge change, however, may affect the various interactive forces between insulin-like growth factor-I (IGF-I) and the binding proteins present in the medium, therefore altering their affinities. In previous studies, PS has been shown to alter apparent IGF-binding activities. Amniotic fluid/Hep G2 IGF-binding protein (IGF-BP-1) was found to contain fewer basic amino acids than the GH-dependent serum IGF-BP-3 (Baxter et al., 1986) and PS was found to reduce IGF binding activity. From other experiments with conditioned media from BRL 3a cells

(Nissley et al., 1977) which are known to contain IGF-BP-2, PS enhanced IGF binding activity.

In our experiments, protamine sulfate enhanced the IGF-I binding to IGF-BPs. From our immunological studies, at least two binding proteins were secreted by the hepatocytes, namely IGF-BP-1 and IGF-BP-2. The increase in IGF-I binding observed with PS may be due to the presence of other IGF-BPs which have a low proportion of basic amino acids. Also differential reaction to PS may be associated with the different degrees of glycosylation found in IGF-BPs.

In previous studies on cultured rat hepatocytes, GH treatment stimulated the accumulation of IGF-I mRNA (Johnston et al., 1989; Norstedt et al., 1987) and the secretion of IGF-I into the medium (Scott et al., 1985a; Scott et al., 1986), but had no effect on IGF-binding protein secretion (Scott et al., 1985b). In contrast, our results show that GH reduced IGF binding activity in the conditioned medium. Our experiments suggest that the binding capacity of the medium was decreased by GH treatment perhaps by inhibition of expression of IGF-BPs.

Glucagon stimulated IGF-I binding activity in our cultures. When combined with GH, it reversed substantially the GH inhibitory effect. This may be due to glucagon's ability of stimulating multiple signal transduction pathways, thereby affecting cellular response due to GH.

#### **4.2 IGF-Binding Protein(s) Characterization**

Greater understanding of the effects of GH and glucagon on the IGF-binding activity present in our media from cultured hepatocytes was possible through further analysis of binding

proteins by Western blotting (WB), dose-dependent inhibition (peptide specificity), and immunological studies.

Conditioned media collected from cells under different treatments were concentrated prior to Western blotting (WB). Initially media were concentrated by lyophilization, which is a fast method for large scale concentrations. However, the pattern of IGF binding activity found in concentrated lyophilized media was much different from that seen with native media. It seemed that during lyophilization and reconstitution, some of the native properties of the binding proteins were lost. This may be a result of conformational changes during reconstitution which can affect the affinity towards the ligand (IGF-I) or complete irreversible denaturation of the ligand binding site(s). Assays on samples concentrated by centricon micro-concentration method revealed results closely comparable to those from non-concentrated samples, therefore this technique was employed in all experiments reported in this thesis.

Western blotting permitted electrophoretic identification of protein subunits that preferentially bound IGFs.  $^{125}$ I-IGF-I binding to the transferred proteins from rat hepatocytes in primary culture resulted in the identification of two bands at  $M_r = 25,000$  and 30-34,000 daltons (Da) (fig.12). The rat liver has been shown previously to secrete IGF-BPs (Schwander et al., 1983; Scott et al., 1985a; 1985b; 1986; Hossenlopp et al., 1987). Our data suggest that the larger molecular weight band perhaps consists of more than one binding protein and parallels Hossenlopp's data. The low molecular weight IGF-BP is similar in size to the one reported from human fibroblasts (Conover et al., 1989) and from human osteoblast-like cells (La Tour et al., 1990). More recently cDNA clones have been isolated from rat liver and have been shown to encode a novel IGF-BP (La Tour et al., 1990). From the deduced amino acid sequence, it was found to be similar to IGF-BP-4, a low molecular weight BP isolated previously from rat and human sera (Donovan et al., 1989).

The binding proteins secreted by the cultured hepatocytes were investigated further using immunological and ligand binding studies.

It had previously been reported that the binding proteins present in media from cultured hepatocytes were similar to the lower molecular weight binding proteins of rat serum (Hardouin et al., 1987). Our Western blot data parallel these findings. IGF-BPs were separated from endogenous IGFs using a Sephadex column and were analysed for both protein content and IGF binding activity. In our studies the protein elution profile paralleled the IGF-binding activity and peaked well before the elution volume of  $^{125}\text{I}$ -IGF-I. This demonstrated that IGF-I binding activity was free of endogenous IGFs (Fig.17).

The IGF-BPs secreted by the cultured hepatocytes showed highly specific binding to IGFs. Even though the IGFs show homology to proinsulin and insulin, the latter did not bind to these BPs. In competitive binding assays the eluted IGF-BPs showed equal affinities for either IGF-I or IGF-II (Fig.20). Previous studies by Hossenlopp et al., reported that IGF-I was slightly more potent than IGF-II when at inhibiting  $^{125}\text{I}$ -IGF-I binding. There appears to be a difference in observed IGF affinities of normal versus acid treated IGF-BPs. Yang et al., have demonstrated differential affinity of unoccupied versus occupied serum IGF-BPs (Yang et al., 1989). They postulated that unoccupied IGF-BPs have a higher affinity for IGF-II. However when acid treated binding proteins were used in competitive binding experiments with either  $^{125}\text{I}$ -IGF-I or  $^{125}\text{I}$ -IGF-II, the apparent affinity for IGF-I was similar to that for IGF-II. Our data agree with theirs and suggest that acid treatment modifies the specificity of unoccupied IGF-BPs. Binoux et al., first suggested that IGF-BPs present in and/or secreted into biological fluids have different specificities for IGFs (Binoux et al., 1982). Most IGF-BPs have equal IGF affinities, but two non-glycosylated low molecular weight binding proteins isolated from cerebrospinal fluid (Hossenlopp et al., 1986) and from MDBK bovine kidney cell line (Szabo et al., 1988) had preferential affinity for IGF-II when using  $^{125}\text{I}$ -IGF-II in competitive binding

assays. Bovine kidney BP has been recognized as IGF-BP-2, our immunological studies (see below) indicate that IGF-BP-2 was present in our conditioned culture media.

Scatchard analyses showed comparable affinity constants for the IGFs with a slightly higher affinity for IGF-II (Fig.21, table 8). Others have demonstrated that IGF-BPs present in rat liver have slightly higher affinity binding constants for IGF-II (Hossenlopp et al., 1987). The observed differences in IGF affinities may suggest that certain IGF-BPs are more abundant than others. Scatchard plots in our studies behave as a two site model. The low affinity binding components may be considered to be non-specific since they represent high concentrations of displacing ligand. For the same reason, when Baxter observed similar Scatchard plot for IGF-BPs present in adult rat serum, he emphasized only the high affinity binding constants (Baxter et al., 1987). In our studies, the similar IGF affinities for IGF-BPs when either radioligand was used for dose-dependent inhibition studies can be rationalized by the following: (i) there are many different IGF-binding proteins that are not well resolved by acid chromatography and co-migrate together, and (ii) among these BPs comprising the large unresolved complex, there is structural heterogeneity resulting in affinity differences for either IGFs. Neither SDS-polyacrylamide gel electrophoresis nor Sephadex G-75 chromatography under acid conditions can resolve accurately IGF-BPs which possess relatively similar molecular weights and therefore this method was only employed to "strip" endogenous IGFs for dose-dependent inhibition studies (also referred to as specificity studies).

Specific antibodies were used to identify the different IGF-BP species secreted by the hepatocytes. Anti-rat IGF-BP-1 and/or anti-rat IGF-BP-2 were found to be immunoreactive with media from our hepatocyte cultures. Immunoprecipitation of IGF-BPs prior to Western blotting was able to resolve individual IGF-BPs. These studies revealed that adult rat hepatocytes secreted both IGF-BP-1 (31-32 kDa) and IGF-BP-2 (35-36 kDa) each contributing to the 30-34 kDa large unresolved band observed by Western blotting (Fig.22). Previous reports demonstrated that both IGF-BP-1 and IGF-BP-2 mRNA are expressed in fetal, neonatal,

and adult liver (Brown et al., 1989). Quantitation of the respective amounts of IGF-BP-1 and IGF-BP-2 present in the hepatocyte media was not feasible. Although BP-2 antibody seemed to be more effective than BP-1, it may be that either: (i) BP-2 is more abundant than BP-1; or (ii) the antibody affinity for BP-2 is greater than the antibody affinity for BP-1. The lower molecular weight complex (25 kDa) was not immunoprecipitated by either antibodies suggesting the presence of another kind of IGF-BP. Since other antibodies directed against either IGF-BP-4 or IGF-BP-3 were not available, we cannot exclude the presence of other binding protein species in the hepatocyte culture media.

### **4.3 Growth Hormone (GH) Effects on IGF-BPs**

An effect of GH on hepatic IGF-BP synthesis and/or secretion has never been clearly demonstrated. Investigators have previously reported that GH has little or no effect on IGF-BP production and/or secretion. The main difficulty has been in maintaining primary hepatocyte cultures for long intervals under serum-free conditions. Serum contains GH, IGFs, glucagon and other growth factors which interfere with set experimental conditions for studying such hormonal effects. Fetal calf serum (FCS), widely used for cell attachment, and cellular growth which has been shown to contain epidermal growth factor (EGF), platelet-derived growth factor (PDGF), and GH which are inducers of IGF-I production (Clemmons et al., 1981; Clemons, 1984).

Because we were able to maintain hepatocytes in culture under serum-free conditions, such studies were possible. Our study demonstrates that GH has a dramatic time-dependent inhibition effect on the secretion of both IGF-BP-1, IGF-BP-2, and the low molecular weight IGF-BP. In contrary, very recent studies on the regulation of mRNA expression of fetal binding protein (IGF-BP-2), increasing concentrations of GH had no apparent effect on BP-2 secretion (Boni-Schnetzler et al., 1990). Therefore, it seems that by keeping hepatocyte cultures

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under serum conditions, the time-dependent inhibitory effect of GH is abolished by GH present in FCS. In our laboratory, we were able to maintain healthy hepatocytes in culture for 48 hours in serum-free medium by replacing fetal calf serum with addition of several co-factors, inorganic elements, and vitamins (see Materials and Methods) before studying hormonal effects on both hepatic IGF-I (Kachra et al., 1991) and IGF-BP production. Our results reflect better the previous *in vivo* studies with streptozotocin (STZ)-treated diabetic rats where insulin therapy restores low hepatic IGF-I mRNA and suppress the elevated hepatic IGF-BP-2 mRNA (Bonì-Schnetzler et al., 1989). Other *in vivo* studies focusing on the effects of growth hormone administration on fasted rats revealed a decrease in hepatic IGF-BP-1 mRNA (Murphy et al., 1991). Possibly these two BPs have well distinct biological roles in the normal vs. diseased state. IGF-BP-1 and IGF-BP-2 mRNA are developmentally regulated since they are expressed in larger amounts during fetal and neonatal life than in adult life (Ooi et al., 1990). Ooi et al. were able to demonstrate a differential induction of IGF-BP-1 and IGF-BP-2 mRNA within different levels of insulin deficiency in diabetic and hypophysectomized rats. They showed that IGF-BP-2 mRNA increased during mild insulin deficiency such as hypophysectomy or fasting whereas IGF-BP-1 mRNA increased only during severe insulin deficiency found in diabetic animals. Therefore they suggested that IGF-BP-1 and IGF-BP-2 may regulate tissue-specific functions during growth (in fetal and neonatal life) and metabolic functions during the normal and diseased catabolic states (in adult life).

From unpublished data in our laboratory (Kachra et al.), there is evidence that GH acts in conjunction with a cAMP-dependent pathway. We observed that cAMP upregulated IGF-I synthesis and secretion but inhibited IGF-BP-1, IGF-BP-2, and low molecular weight BP synthesis and secretion. The fact that GH inhibits hepatic IGF-BP secretion but stimulates IGF-I mRNA and IGF-I secretion fits well with the earlier postulated somatomedin theory that IGFs are the main mediators of GH action and a decrease in IGF-BP secretion correlates well with an increase in IGF-I biological activity (Solomon and Daughaday, 1957).

#### **4.4 Glucagon Effects on IGF-BPs**

Upon treatment with glucagon, both 25 and 30-34 kDa bands were revealed by Western blots suggesting that glucagon stimulates IGF-BP production. From densitometric data obtained from these blots, glucagon differentially enhanced the IGF-BP(s) secreted into the conditioned medium. Glucagon augmented the production of the lower molecular weight IGF-BP and the 30-34 kDa BP(s) (see figs.13 and 14).

From previous studies on the regulation of GH-dependent IGF-BP-1 in fetal liver explants, glucagon was reported to stimulate by 90% IGF-BP-1 production (Lewitt et al., 1989). He suggested that agents capable of stimulating IGF-BP-1 production also can augment cAMP levels. Other groups have previously demonstrated that many of the trophic hormones are known stimulators of IGF-I production (Bucher et al., 1975; Seneviratne et al., 1990; Orłowski et al., 1990). This effect is associated with concomitant cAMP production which augments IGF-I production (Tode et al., 1989). Since glucagon has been known to stimulate both inositol triphosphate (IP<sub>3</sub>) and cAMP in hepatocytes (Wakelam et al., 1986), the production of IGF-I might be a result of elevated cAMP levels. Therefore cAMP-dependent mechanisms might also modulate both IGF-I and IGF-BP-1 production.

Other *in vivo* studies by Boni-Schnetzler et al. postulated that insulin stimulated GH secretion which in turn increased IGF-I and decreased IGF-BP production. Glucagon antagonizes insulin's actions perhaps by an augmentation of hepatic IGF-BPs. Our data supported this finding since glucagon stimulated IGF-I mRNA (Kachra et al., 1991) and hepatic IGF-BPs. Since IGF-BPs, in the size range secreted by the hepatocytes have previously been shown to potentiate IGF binding and action (Elgin et al., 1987), it is possible that glucagon exerts its hepatotrophic effect through an augmentation of IGF-BP secretion.

Lewitt et al., showed that IGF-BP-1 production depends on intracellular glucose availability. Therefore, it is possible that both insulin and glucagon can regulate short term metabolic activities by regulating IGF-BP mRNA accumulation and/or secretion.

#### **4.5 Combined Effects of GH and Glucagon on IGF-BPs**

The most striking effect was observed when GH was co-incubated with glucagon for treatment. The inhibitory effect of GH was reversed by glucagon's presence (fig.13). GH and glucagon when incubated separately stimulate equipotently IGF-I gene expression and IGF-I production. When co-incubated, there was a 10-fold stimulation in IGF-I mRNA accumulation but not in IGF-I production. It was concluded from this that a disproportionate effect may be due to generation of poorly translated mRNA species (Kachra et al., 1991).

From Western blots and densitometric analysis, both IGF-BPs were augmented by glucagon's presence. When compared to control (non-treated), an additive effect was seen for the low IGF-BP whereas the 30-34 kDa complex was slightly increased. This suggested that the low molecular weight BP is different from the other IGF-BPs in the 30-34 kDa complex and differently regulated by these hormones.

Our hepatocyte cultures appear to reflect the *in vivo* hypophysectomized state. Thus GH levels drop acutely after hypophysectomy and a corresponding increase in hepatic IGF-BP levels has been described (Barenton et al., 1988). Similar results were reported by Margot et al. where a low molecular weight rat fetal IGF-BP was greatly augmented after hypophysectomy (Margot et al., 1989).

Perhaps the degree of cellular IGF actions mediated by IGF-I is under endocrine control. One can suggest an interplay between GH and IGF levels, the concentration of both locally produced IGF-BPs and IGF-BPs present in the circulation affecting overall growth and

development. Factors which can stimulate or antagonize IGF actions may affect at the central nervous system (CNS) level (i.e neurotransmitters, excitatory or inhibitory neuropeptides, and locally produced peptides exert either positive or negative feedback on GH production), or at other possible peripheral sites where IGFs and IGF-BP are synthesized and secreted (i.e cAMP levels, insulin which affects GH levels).

Recently IGF-BP-1 was recognized to possess hydrophobic properties and appears able to cross capillary walls (Bar et al., 1990). Other investigators have shown that IGF-BP-1 and the type I IGF receptor compete for IGF-I (Rutanen et al., 1988; Ross et al., 1989). Our laboratory has previously shown that liver membranes lack type I IGF receptor (Barenton et al., 1987). Therefore IGF-I action on rat liver may occur through IGF-I-BP complex translocation in the hepatocytic cells.

Other studies using IGF-I analogs have demonstrated that the ability of IGF-I to stimulate DNA synthesis and BP secretion depends on the affinity of IGF-I for both type I receptor and binding proteins (Clemmons et al., 1990). This suggests that IGF-I actions are not only exerted through the receptor's tyrosine kinase cell signalling pathway.

It is still very difficult to assign specific functions for each individual IGF-BP identified and characterized. The progress in this field however is fast and understanding the role(s) of the different IGF-BPs seems within reach which should lead to a fuller understanding of how the IGFs act and are regulated in their diverse anabolic, mitogenic, and differentiative roles. IGFs are involved in various agricultural and clinical applications. They range from stimulation of animal growth to wound healing after surgery, therefore the greater understanding of their roles and actions will provide a better health and welfare in the human and animal race.

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