DEVELOPMENT OF A TECHNIQUE FOR MEASURING INSULIN-LIKE GROWTH FACTOR-I IN SWINE: APPLICATION TO THE STUDY OF THE IN VIVO AND IN VITRO EFFECTS OF GROWTH HORMONE IN NEONATAL PIGS

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(ABSTRACT)

The relationship between porcine growth hormone (pGH) and insulin-like growth factor-I (IGF-I) in swine was investigated. IGF-I levels were measured by radioimmunoassay (RIA) with an antisera specific for human IGF-I after ultrafiltration of acidified samples. Ultrafiltration quantitatively separated carrier proteins and IGF-I. Failure to separate these species interfered with the RIA. Using this assay, swine chronically treated with pGH had 2.6-fold higher sera levels of IGF-I than controls, whereas, serum IGF-I from a hypophysectomized animal was only 4% that of normal animals.

The ultrafiltration procedure was incorporated into a protocol to measure IGF-I in sera from neonatal swine treated with pGH (50 $ug \cdot kg\ BWt^{-1} \cdot d^{-1}$). Treatment of neonatal pigs with pGH for one or two weeks elevated pGH in sera, but did not significantly affect either growth or serum IGF-I concentrations. Preliminary studies were performed to determine if neonatal swine hepatocytes secreted

IGF-I *in vitro*. Results indicated that neonatal hepatocytes synthesized limited quantities of IGF-I that approached the detection limits of the RIA. Furthermore, pGH did not stimulate hepatocyte IGF-I synthesis *in vitro* or *in vivo*. Neonatal hepatocytes synthesized a protein species that bound labeled IGF-I and had a molecular weight similar to a carrier protein in swine serum. Estimation of the rate of carrier protein synthesis suggested that IGF-I and carrier proteins are coordinately regulated in isolated neonatal hepatocytes. These results suggest that IGF-I is not inducible in the neonatal pig by GH therapy and that growth in neonatal swine is either maximal or GH-independent.

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DEDICATION

I dedicate this work to the bravest member of my family: my sister, Nancy. Wherever you go may God be with you.

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CHAPTER 1.

LITERATURE REVIEW

Consumption of red meat by Americans has declined steadily during the past fifteen years. A major impetus for reduced consumption has been the repeated warnings from the biomedical community that consumption of diets containing high levels of saturated fats increases the risk for heart disease and cancer. Despite the decreased per capita demand, the production of beef and pork has increased about 8% in the past fifteen years. This increase has been due to an expansion of population and of export markets. Despite increased market size, the altered buying habits of the public have markedly reduced profits for producers. Obviously, livestock producers must develop novel strategies for improving the efficiency of production. Since the current gross efficiency of meat animal production (units of edible product marketed/units of feed input) is 17%, any method that will increase this value, such as decreased carcass fat content, will be beneficial to the livestock industry.

The general goal of our research group concerns improving the efficiency of swine production. One of the approaches employed is elucidation of the endocrine regulation of swine growth. Such information will provide the basis for developing specific strategies to improve the efficiency of growth. Recent results demonstrating that the administration of porcine growth hormone (GH) to rapidly growing swine improved growth performance (1-3) have shown that the rationale for treatment with growth factors is indeed sound. While the mechanism of action of porcine GH (pGH) is not fully understood,

it is currently believed that at least some of its *in vivo* effects are mediated by the somatomedins/insulin-like growth factors (IGFs).

Reviews concerning the chemistry, biological actions and the characteristics of cell surface receptors for GH (4-6) and IGFs (7-14) are available. The initial discovery of the growth promoting effects of GH was made indirectly in 1921 by Evans and Long (15). They observed accelerated growth rates in rats repeatedly injected with emulsions of the anterior pituitary gland. In 1944, Li and Evans (16) purified this growth promoting substance from bovine pituitary glands. Subsequently, GH was found to be required for normal growth in most mammalian species. The lack of GH, found clinically in hypopituitary patients or induced experimentally by hypophysectomy (HYPOX), is associated with a retardation of growth processes. The administration of GH to HYPOX animals or hypopituitary patients accelerates somatic growth as measured by increases in body weight and longitudinal bone mass. The hypersecretion of GH by the pituitary results in a condition known as acromegaly, where the bones of the face and extremities are notably enlarged in addition to accentuated long bone growth.

The dependency of growth on GH is developmentally regulated. Reid (17) reported that congenital absence of the pituitary gland in man did not decrease body length or weight at birth. In addition, HYPOX of monkeys (18) or decapitation of rabbits *in utero* (19) has no

effect on fetal growth. The ontogeny of GH dependent growth in rats was investigated by Walker and coworkers (20). In these studies, growth was relatively unaffected in HYPOX rats less than 10 d old. Between 10-28 d of age, removal of the pituitary gradually decreased growth as measured by body weight. Body weight remained constant in HYPOX rats older than 28 d. Some investigators (21,22) have correlated this gradual increase in responsiveness to GH of animals during the postnatal period to changes in tissue binding sites for GH. Thus, the binding of GH to liver membranes prepared from rats (21) and sheep (22) was low in fetal and neonatal animals and gradually increased with age.

The biological effects of GH are diverse and in some cases contradictory. These effects can be 1) acute insulin-like; 2) chronic insulin antagonistic; and 3) mitogenic, affecting cellular differentiation (4). Because of this wide range of effects, it has been suggested that variants of GH, or multiple forms of the GH receptor, or a combination of both, mediate these various biological effects (5,6). The possibility that GH may be acting indirectly in some target cells has also been proposed (7-10). The lack of a direct effect of GH in many in vitro systems has made it difficult to define an in vivo biological response that is mediated by GH (6).

A major result of treatment of a HYPOX animal with GH is the stimulation of longitudinal bone growth. In 1957, Salmon and Daughaday made an important observation (23). They were examining

^{35}S-sulfate incorporation into proteoglycans by rat cartilage explants. They observed that serum from normal rats stimulated proteoglycan synthesis, while serum from HYPOX rats did not. When GH was administered to HYPOX rats, the resultant serum also stimulated ^{35}S-sulfate incorporation by cartilage explants. However, direct addition of GH to the cultures failed to stimulate ^{35}S-sulfate incorporation. They proposed the existence of "sulfation factors", which mediated the effect of GH on cartilage metabolism. These factors were later given the name "somatomedins" (24). Subsequent characterization of the somatomedins led to the hypothesis that GH affects skeletal growth indirectly. In this theory, GH derived from the pituitary stimulates the liver to synthesize somatomedins which are then secreted into the circulation. The somatomedins act directly on target cells (in this case chondrocytes) to stimulate growth.

Isaksson and coworkers have attempted to show that GH affects bone growth directly. These investigators have found that injection of GH locally into the epiphyseal growth plate (the site where cellular proliferation of chondrocytes is occurring) of HYPOX rats stimulates bone growth (25). Furthermore, they have shown that specific binding sites for GH are present on chondrocytes isolated from rabbit ear and epiphyseal plates (26). However, the specific binding of GH is much lower than the specific binding of somatomedins when the data is expressed per unit of DNA (27). In addition, the

administration of somatomedins to HYPOX rats in the absence of GH stimulated growth in the epiphyseal plate (28). Clearly, the question of whether GH has a direct or indirect mechanism of action in vivo has not been resolved.

The somatomedins, or more broadly IGFs[1], were independently discovered by two other groups. Studies performed in the laboratory of Froesch and co-workers revealed that approximately 90% of the insulin-like effect of serum on glucose metabolism by adipose tissue in vitro was not suppressed by specific antisera to insulin (29). They called these factors "non-suppressible insulin-like activity". After further characterization, this activity was found to be composed of primarily two polypeptides which were renamed insulin-like growth factors I and II (30,31). This nomenclature is most widely accepted and will be used throughout this dissertation.

The third discovery was made by Temin and coworkers (32) as a result of purifing growth factors in serum that were required for the proliferation of cells in vitro. They partially purified a polypeptide fraction from calf serum that stimulated the proliferation of chick fibroblasts in culture. They later found that similar peptides were produced by a Buffalo rat liver cell line (BRL-3A) in culture (33,34). They named these polypeptides

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[1]The name somatomedin implys that the peptide is dependent on GH. This classification is now regarded as too narrow because the hormones from this family vary in their GH dependence (see text).

"multiplication-stimulating activity" based on their ability to stimulate the proliferation of certain cells of mesodermal origin.

It is now recognized that all three peptides (somatomedins, insulin-like growth factors, and multiplication-stimulating activity) belong to a family of polypeptide hormones having similar properties, viz., 1) similar molecular weights (7-10 kDa), 2) structural and functional similarities to insulin, and, 3) mitogenic activity for a wide variety of cells in culture. This family can be subdivided into two general categories based on receptor reactivity and immunological properties viz., IGF-I-like peptides with a basic pI (8.0-8.5) and IGF-II-like peptides with an acidic pI (6.0-6.5). IGF-I isolated from human serum is identical to somatomedin-C (35) and is under regulation by GH. Thus, serum levels of IGF-I are characteristically depressed in hypopituitary patients and elevated in acromegalics (36-38). IGF-I is believed to be important in the regulation of postnatal growth, in part, because serum levels are low at birth and gradually increase to peak levels at puberty (36,38). Thereafter, the levels of IGF-I slowly decline. In contrast, IGF-II-like peptides appear to be involved in regulating fetal growth (39). The levels of IGF-II are high in the fetal circulation of a number of species and decline postnatally (40-43). Recently, Graham et al. (44) have used a cDNA probe for rat IGF-II to show that the mRNA for rat IGF-II is present in livers from neonatal rats, but absent in livers from older rats. IGF-II-like peptides are more insulin-like

and less GH dependent than IGF-I-like peptides (36). Thus, Zapf et al. (36) found that the levels of IGF-II in the sera of acromegalic patients were not different than normals, whereas, in hypopituitary patients, the levels of IGF-II were lower.

The biological responses that are elicited by the IGFs and insulin are essentially the same. These include effects on glucose and amino acid metabolism, as well as effects on protein and DNA synthesis. Distinct receptors for the IGFs are present on most cell types (for review see 11-13). These receptors have been classified as type I or type II based on their specificities. Type I receptors show structural and functional similarities to the insulin receptor and have the following binding preference: IGF-I>IGF-II>>insulin. Type II receptors are not related to the insulin receptor and do not bind insulin. They preferentially bind IGF-II over IGF-I. Both IGF-I and IGF-II are capable of binding to the insulin receptor. Because IGF receptors and insulin receptors are usually present on the same target cell, the task of assigning a particular biological response to binding to one of these receptors is difficult. The receptor involved in a response is deduced from experiments designed to determine the relative potencies of the ligands (IGFs and insulin). For example, if the concentration of insulin required to elicit a response is 50-100 times lower than the concentration of IGFs required for the same response, then it is assumed that binding of insulin to the insulin receptor is the mediator of the response.

This can be confirmed by using Fab[2] fragments prepared from anti-insulin receptor IgG to block binding of the ligand to the insulin receptor (45). Similar probes are not yet widely available for the IGF receptors.

When IGF receptors were initially characterized, it was assumed that the IGFs were stimulating classical insulin responses (stimulation of glucose or amino acid transport) by cross-reaction with the insulin receptor and that insulin was stimulating classical growth responses (stimulation of DNA and protein synthesis) by cross reaction with the IGF receptors. Although this assumption was subsequently shown to be the case in some cell types (i.e., adipocytes), it is not always so (11). The specific responses that each receptor mediates must be determined for each cell type examined.

Although there are many similarities between the IGFs and insulin, there is one important and striking difference. Insulin circulates in blood as the free peptide and its levels are subject to wide daily variations depending on the nutritional and physiological status of the animal. In contrast, the IGFs circulate in blood bound to specific carrier proteins (46). Their serum levels are regulated over longer periods of time requiring several hours to days to be

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[2]The intact divalent ant-ireceptor IgG inhibits insulin binding to adipocytes, however, it also stimulates glucose oxidation. Monovalent Fab fragments prepared from this antibody will inhibit insulin binding but do not stimulate glucose oxidation.

altered. Thus, serum IGF-I levels begin to rise 6-8 h after GH treatment of hypopituitary children and do not reach peak levels until 16-28 h (47). At least two forms of serum carrier proteins exist. A large molecular weight form (150-200 kDa) which contains most of the endogenous IGF-I in normal adult animals (47-49), is acid labile (50) and is regulated by GH (51-54). This form is believed to be made up of subunits (50,54). According to one model, the complex is made up of at least two subunits, viz., 1) an acid stable subunit (50-60 kDa) that retains IGF binding activity and 2) an acid labile subunit (45-60 kDa) that does not bind IGF (50). Another model proposes that the complex is made up of six acid stable subunits (22-25 kDa) each of which retains IGF binding activity (54). A lower molecular weight carrier protein (30-45 kDa) is also present in plasma. This species is acid stable (55), appears to be inversely regulated by GH (55,56), and accounts for most of the unsaturated and/or exchangeable carrier protein binding sites in serum (55). This protein may be part of the 150 kDa complex, however it is immunologically distinct from the 50-60 kDa acid stable component of the large complex (52). Although, specific physiological roles for the carrier proteins have not been clearly defined, it appears that they extend the half-life of IGFs in blood (57). In addition, Zapf et al. (58) reported that a crude preparation of carrier proteins totally abolished the insulin-like effects of the IGFs on isolated rat adipose cells without affecting the actions of insulin. Based on

this observation, it is currently believed that the IGFs are not active when bound to the carrier proteins and must be dissociated in order to stimulate target cells. The finding that total circulating levels of IGFs are about 50-200 times greater than the concentrations required to elicit a response in target cells supports this hypothesis. Therefore, the carrier proteins offer another level of control beyond the normal and accepted hormone-receptor-postreceptor translation hypothesis (59). Target cells must not only be able to respond to the IGFs (i.e., possess receptors), but they also must be able to release the IGFs from their carrier proteins. The mechanism by which the IGFs are released from their carrier proteins and subsequently delivered to the target cells is currently unknown.

Several investigators have reported that the carrier proteins interfere with measurement of serum IGF-I by radioimmunoassay (see chapter 2 for discussion). Chatelain et al. (60) found that incubation of serum at neutral pH increased the level of immunoreactive IGF-I in serum without increasing the amount of free IGF-I as measured by gel permeation chromatography. This process was time-, temperature-, and pH-dependent. Moreover, this increase was inhibited by a wide range of protease inhibitors and chelating agents. Clemmons et al. (61) found that the release of IGF-I from carrier proteins was promoted by addition of heparin to serum. This process was also shown to be dependent on proteolytic activity. These investigators suggested that proteolytic enzymes and

heparin-like substances might play a role in IGF-I action *in vivo* by regulating the release of this hormone from carrier proteins.

A number of studies have shown that the liver is an important site for synthesis of IGFs. The perfused liver model (62-64), liver organ culture (65), and primary hepatocytes in culture (66-69) have all been shown to produce IGFs. In addition, IGFs are produced by other cultured cells including: fibroblasts (70), smooth muscle cells (71), and ovarian granulosa cells (72). The finding that many tissues are capable of synthesizing IGFs has led to the proposal that these peptides act through autocrine or paracrine mechanisms on target tissues (73). Furthermore, D'Ercole et al. (74) have proposed that measurement of the IGFs in tissues may be more meaningful than those for serum.

The objective of this research was to investigate the relationship(s) between GH and IGFs in the neonatal pig. Since some of the *in vivo* effects of GH are mediated by the IGFs, a method for assessing IGF levels in the neonatal pig was required. IGF-I levels were measured because this peptide is more tightly regulated by GH than IGF-II. A method was validated for determining the concentration of IGF-I in swine serum (chapter two).

While treatment of rapidly growing swine with pGH improves growth performance (1-3), the effects of exogenous pGH on the growth of neonatal pigs has not been previously investigated. Since the only route of administration of pGH has been by injection, treatment of

neonatal pigs would be more cost effective than similar treatment of rapidly growing pigs. The task of administering pGH daily to 50-100 kg pigs housed in a large production environment is formidable. Moreover, smaller doses of pGH may be required to treat neonatal pigs. Consequently, possible effects of pGH on serum levels of IGF-I and growth rates of neonatal swine were investigated (chapter three). Finally, primary cultures of neonatal swine hepatocytes were prepared to examine direct effects of pGH on IGF-I synthesis (chapter three).

CHAPTER 2.

VALIDATION OF A PROCEDURE FOR MEASURING IGF-I IN SWINE SERA

INTRODUCTION

A variety of methods have been developed to quantify IGFs in serum (for review see 75). Initially, IGFs were quantified by their various biological activities. In these bioassays, the incorporation of ^{35}S-sulfate into proteoglycans of cartilage, the incorporation of ^{3}H-thymidine into DNA of fibroblasts, and the stimulation of net gas exchange, glucose uptake, or lipid synthesis in fat cells were measured. Bioassays estimate the combined IGF-I and IGF-II content of serum. The binding proteins affect these bioassays in different ways. For instance, the binding proteins completely interfere with IGF activity in the fat cell assay, but do not affect IGF activity in the cartilage bioassay (58,75). In addition, other factors in serum, such as the presence of inhibitors or stimulators of the activities being measured, may affect the results of bioassays. Although bioassays may reflect the *in vivo* physiological situation more accurately, they fail to distinguish between the specific factors contributing to the overall IGF activity of serum.

When purified IGF-I and IGF-II became available for radiotracer preparation with ^{125}I, a number of radioligand binding assays were developed for measuring IGFs in serum (75). These radioligand assays can be divided into three types: 1) radioreceptor assays (RRAs), 2) competitive protein binding assays (CPBAs), and 3) radioimmunoassays (RIAs). All three types of assays are largely unaffected by the unrelated inhibitors and stimulators and thereby have advantages

over bioassays. However, each assay is affected by the presence of specific carrier proteins in serum. In fact, CPBAs use a partially purified preparation of carrier proteins as the "acceptor" in the assay system (76). Therefore, the samples must be treated to completely remove endogenous binding proteins before the sample can be assayed in a CPBA.

Various tissues contain receptors for IGF-I and along with the insulin receptor these form the basis for the RRA (75). Depending on the labeled peptide used (IGF-I or IGF-II) and the tissue source of the membranes, the specificity of these assays varies. Human placental membranes have been used to assay IGF-I (77); however, this receptor system shows considerable cross-reactivity with IGF-II (75). Overall, most RRA systems are not entirely specific for either IGF-I or IGF-II. Rat liver or placental membranes are exceptions that seem to be suitable matrices for quantifying IGF-II (75). Serum carrier proteins also interfere in these assays, thereby necessitating sample treatment prior to assay (75).

RIA is the only method currently available for the specific distinction of IGF-I and IGF-II. Specific RIAs for IGF-I have been developed in several laboratories (36-38,78). However, serum carrier proteins also interfere in all RIAs for IGF-I. Furlanetto et al. (38) reduced interference from human carrier proteins by utilizing a nonequilibrium RIA technique in which the sample was incubated with the first antibody for 72 h prior to the addition of labeled

antigen. The efficacy of this technique for IGF-I measurement in untreated human serum is controversial (79,60). Zapf et al. (36) separated IGF-I from carrier proteins by passing samples over Sephadex G-50 that had been equilibrated in 1M acetic acid before assay by RIA. Bala and coworkers reported that acidification and subsequent lyophilization of serum was also effective in eliminating carrier protein interference in their RIA for IGF-I (78). Other investigators have acidified and incubated serum for 48 h at 37°C before RIA of IGF-I (80). Daughaday et al. (81) developed an acid-ethanol extraction procedure that gave quantitative recovery of IGF-I from serum. The apparent advantage of these methods is that physical separation of IGF-I from carrier proteins does not seem to be necessary prior to RIA. Some investigators have suggested that the carrier proteins are irreversibly denatured by acid treatment and that their affinity for IGF-I is decreased compared to the antibodies used in the RIAs (78,80,81). While such alterations may occur, there is evidence that the residual specific IGF-I binding activity present after acidification and neutralization of serum samples affects the results of the IGF-I RIA (36). Additionally, in all reports human or rat sera were used as a test matrix and the possibility exists that species differ in carrier protein affinity for IGF-I.

The objective of this research was to investigate the possibility of using a rabbit antisera specific for human IGF-I to measure serum levels of swine IGF-I. The specificity of this antiserum, as well as

its use to measure IGF-I in the sera of various species, has been previously reported (38,48,80,82,83). We have used this antisera to measure IGF-I levels in swine sera (1,2,84) that had been pretreated with heparin as outlined by Clemmons et al. (61). Heparin apparently liberates IGF-I from the carrier proteins by a mechanism dependent upon proteolytic activity at neutral pH. However, recovery of IGF-I from swine serum was not quantitative using this method. The effectiveness of some of the pretreatment methods described above for dissociation of IGF-I from carrier proteins was examined. With the exception of initial acidification of serum samples followed by chromatographic separation of IGF-I and carrier proteins under acidic conditions, tested methods were confounded by the continued presence of the swine serum carrier proteins for IGF-I. A method based on ultrafiltration of treated serum samples was investigated as an alternative to gel filtration chromatography.

MATERIALS AND METHODS

Preparation of pooled sera. Blood (400 ml) was obtained by puncture of the vena cava of four barrows that weighed approximately 50 kg. Blood was coagulated at 4°C for 16 h, and centrifuged at 1800xg for 30 minutes. The serum (200 ml) was pooled and stored as aliquots at -70°C prior to use. This material is referred to as "pooled sera".

IGF-I radioimmunoassay for swine serum samples. IGF-I antisera raised against highly purified human IGF-I was generously provided by Dr. L.E. Underwood through the National Hormone and Pituitary Program. Human recombinant IGF-I (h_rIGF-I; Amgen Biologicals, Thousand Oaks, CA) was used for standard and preparation of labeled antigen. This peptide was iodinated by the solid phase iodogen method (85). Iodogen (1,3,4,6- tetrachloro-3 ,6 -diphenylglycoluril, Pierce Chemical Co., Rockford, IL) was dissolved in $CHCl_3$ at a concentration of 1 mg/ml. A fresh stock solution was made for each iodination and was diluted 1:50 in $CHCl_3$ immediately prior to use. Glass reaction vials were acid washed and sequentially rinsed in deionized water and $CHCl_3$. The vials were dried in an oven (60°C) and stored dessicated until use. A vial was coated with iodogen (1 ug) by addition of 50 ul of the diluted stock solution and subsequent evaporation of the $CHCl_3$ under a stream of nitrogen. One microgram of h_rIGF-I dissolved in 20 ul of 0.1 M acetic acid (manufacturer's storage recommendations) was added to the vial along

with 75 ul of 0.5 M Na phosphate buffer, pH 7.4 (final pH 6.8). The vial was immediately capped and 0.5 mCi (5 ul) of $Na^{125}I$ (NEN Research Products, Boston, MA) was injected through the septum. The reaction proceeded for 10 minutes and was stopped by transferring the vial contents to a column (1.5x30 cm) containing Sephadex G-50 (fine) equilibrated with 50 mM Tris-HCl, 0.01% (w/v) thimersol, pH 7.4 (buffer A). Column chromatography was used to separate free and protein bound ^{125}I. The sample was eluted with buffer A containing 0.001% (w/v) phenol red (buffer B) at a flow rate of 24 ml/h. One ml fractions were collected in polypropylene tubes containing one ml of buffer B with 0.5% (w/v) bovine serum albumin (BSA; Bovuminar^{R}; Armour Pharmaceuticals, Kankakee, IL; buffer C). Fractions containing protein-bound ^{125}I eluting after void volume but before phenol red were pooled and diluted to $1.5x10^{6}$ cpm/ml with buffer C. The radioactivity precipitated by 5% (w/v) trichloroacetic acid (TCA) in this pool averaged greater than 90% of the total radioactivity added. The ^{125}I labeled $h_{I}IGF$-I was stored at $4^{o}C$ for a maximum of 8 weeks. When the quantity of ^{125}I precipitated by the addition of TCA was 80%, material was further purified by Biogel AG 1-X8 (Biorad, Bethesda, MD) ion exchange chromatography. The specific activity of this material, as determined by self displacement in the radioimmunoassay, ranged from 100 to 400 uCi/ug (average 300 uCi/ug). This material was used for all experiments requiring ^{125}I labeled $h_{I}IGF$-I.

Radioimmunoassay (RIA) was performed in 12x75mm polystyrene culture tubes (Sarstedt, Princeton, NJ) utilizing a buffer system consisting of 50 mM Tris-HCl, 10 mM EDTA (ethylenediaminetetra-acetic acid), 0.25% (w/v) BSA, 0.2% (v/v) Tween 20, 0.02% (w/v) protamine sulfate, and 0.01% (w/v) thimersol, pH 7.4 (RIA buffer). The release of IGF-I from carrier proteins as a result of exposure to glycylglycine-HCl (glygly), heparin, and acid ethanol were compared as described below. Since the carrier proteins interfered in the antibody-antigen reaction of the RIA, a method was developed for initial release and subsequent separation of IGF-I from carrier proteins.

The method of Chatelain et al. (60) was utilized for glygly treatment. Briefly, aliquots of serum (100 ul) were incubated in 0.2 M glycylglycine-HCl, pH 2.0 (120 ul, final pH 3.5) for 48 h at room temperature in polypropylene screw cap tubes. Incubation times of shorter or longer periods gave lower values of IGF-I in the RIA. The sample was then diluted in RIA buffer (final dilution 1:40-1:100) and 50 ul aliquots were assayed in the IGF-I RIA. The final pH of the sample prior to assay was 7.2 to 7.4.

Heparin treatment was adapted from Clemmons et al. (61). Aliquots of serum (100 ul) were incubated in buffer B (400 ul) containing 10 U/ml heparin (Sigma Chemical Co., St. Louis, MO) for 48 h at 37^{o}C. Longer or shorter incubation times gave lower relative values in the IGF-I RIA. Sample dilution and assay were performed as

described for glygly treatment.

Acid ethanol treatment was performed as described by Daughaday et al. (81). Briefly, serum (200 ul) was added to an acid ethanol mixture (87.5% absolute ethanol/12.5% 2 N HCl (v/v); 800 ul) and incubated for 2 h at room temperature. The sample was centrifuged at 1800xg (swinging bucket rotor) for 0.5 h and 500 ul of supernatant was removed and neutralized with 200 ul 0.855 M Tris base (unadjusted pH). The sample was diluted in RIA buffer (final dilution 1:40-1:100) and 50 ul aliquots were assayed in the IGF-I RIA. Volumes of neutralized acid ethanol equivalent to the amount present in a 1:40 dilution of sample were added to tubes containing known amounts of h_rIGF-I to assess possible interference of these substances in the RIA.

In the IGF-I RIA, diluted samples (50 ul) derived from one of the above treatments or ultrafiltration were added to 350 ul of RIA buffer containing first antibody at a final dilution of 1:8000. All tubes were vortexed and incubated for 16 h at 4°C (non-equilibrium assay conditions). Then ^{125}I-h_rIGF-I (1.5×10^6 cpm/ml) was diluted 1:10 in RIA buffer and 100 ul was added to each tube. The tubes were vortexed and incubated 24 h at 4°C. The antibody-antigen complex was precipitated by the addition of goat anti-rabbit sera (0.8 ml, Research Products International Corp., Mt. Prospect, IL) added at a concentration of 0.005% (v/v) in RIA buffer supplemented with 6% (w/v) polyethylene glycol. The tubes were

incubated for 1.5 h at 4°C. Normal rabbit carrier serum (1:50 in RIA buffer, 100 ul) was added and tubes were incubated for 1.5 h at 4°C after which they were centrifuged at 1800xg for 0.5 h. The supernatants were aspirated and the radioactivity associated with the antigen-antibody pellet was measured in a LKB model 1274 RIAGAMMA^{R} counter. The radioactivity precipitated by the goat anti-rabbit sera/polyethylene glycol mixture in the absence of first antibody averaged 1-2% of the total radioactivity added.

Chromatographic analysis of free vs. bound ^{125}I-h$_r$IGF-I. One ml of pooled sera and one ml of ^{125}I-h$_r$IGF-I (1x10^6 cpm/ml) were mixed and incubated for 16 h at 4°C. This sample was either chromatographed immediately (see below) or treated with one of the following: 1) glycylglycine-HCl (32), 2) heparin (33), or, 3) acid ethanol (64), followed by chromatography. Details of the treatment protocols are outlined in figure 1.

Immediately prior to chromatography, sucrose was added to each sample to a final molarity of 0.47. The sample was applied to a column (2.5x90cm) containing Sephacryl S-200 (Pharmacia Fine Chemicals, Piscataway, NJ) that had been equilibrated in 50 mM Tris-HCl, 0.2 M NaCl, 0.01% (w/v) thimersol, pH 7.4. Sample was eluted in an ascending manner with equilibration buffer at a flow rate of 30 ml/h. Four ml fractions were collected. The eluate was monitored for absorbance at 280 nm and ^{125}I in each fraction was

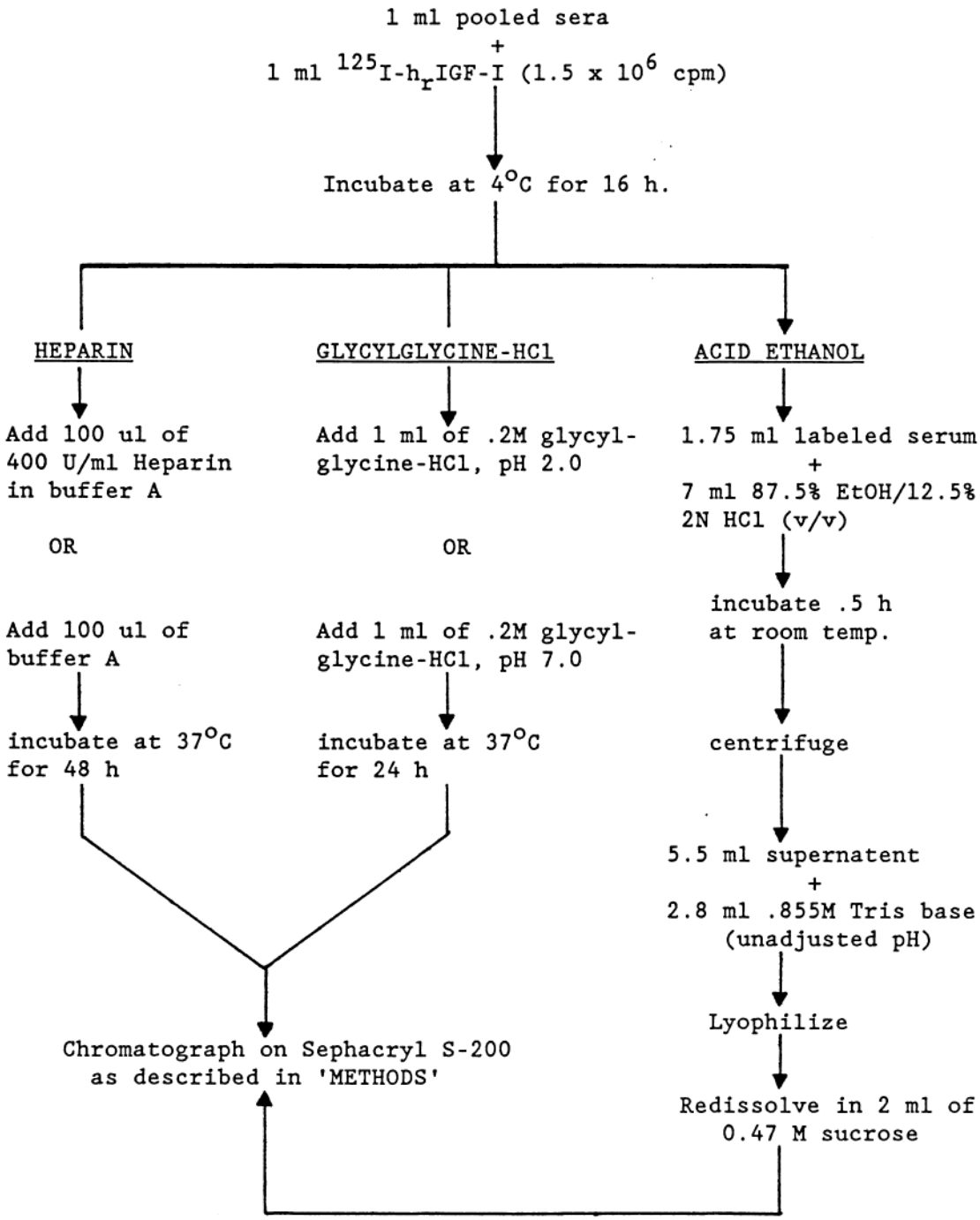

Figure 1. Outline of treatment protocols used on the labeled pooled sera prior to Sephacryl S-200 gel permeation chromatography.

measured in a Packard Model 5230 Auto Gamma counter. The elution volumes were converted to K_d values based on the elution volume of blue dextran (165 ml) and potassium dichromate (470 ml). The column containing Sephacryl S-200 was standardized using the following proteins: aldolase (158 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen (25 kDa), ribonuclease A (13.7 kDa) [Pharmacia Fine Chemicals, Piscataway, NJ], and ^{125}I-h_rIGF-I (7.85 kDa) [figure 2].

In other experiments, the pooled sera was subjected to Sephadex G-50 gel filtration chromatography. The objective was to determine whether serum carrier proteins retained the ability to bind ^{125}I-h_rIGF-I after serum had been treated with acid, neutralized, and then diluted 1:80 (a typical sample dilution for the IGF-I RIA). Initially, ^{125}I-h_rIGF-I ($3.0x10^5$ cpm; 200 ul) was incubated with serum (75 ul) for 16 h at 4°C. One half of the sample was added to 3 ml of 3.5 M acetic acid, while the other half was added to 3 ml of buffer A. Samples were incubated for 2 h at room temperature and then concentrated to dryness using a Savant Speed Vac^R Concentrator (Hicksville, NY). Concentration was done overnight with sample chamber heater and heat lamp off. Samples were dissolved in 3 ml of buffer A containing 0.47 M sucrose and incubated overnight at 4°C. One ml aliquots were applied to columns (1.5x30cm) containing Sephadex G-50 that had been equilibrated in 50 mM Tris-HCl, 0.1 M NaCl, 0.01% (w/v) thimersol, pH 7.4 (buffer D). The samples were

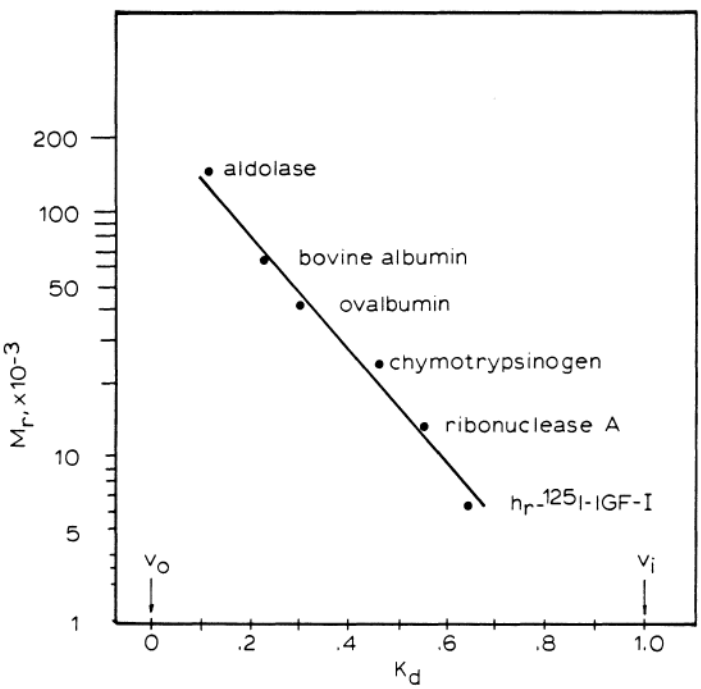

Figure 2. Standardization of Sephacryl S-200 gel filtration chromatography column by elution of proteins of known molecular weight. All samples were applied in 2 ml of elution buffer containing 0.47 M sucrose. The column was standardized with 4 separate fractionations: 1) blue dextran & potassium dichromate, 2) aldolase, ovalbumin, and ribonuclease A, 3) bovine albumin & chymotrypsinogen, and 4) ^{125}I-h_rIGF-I (1x10^6 cpm).

eluted with buffer D at a flow rate of 25 ml/h. One ml fractions were collected and ^{125}I content of each fraction was measured. Radioactivity routinely eluted in three distinct regions. Region I contained radioactivity that coeluted with blue dextran at V_o and was defined as "bound". Region II included ^{125}I that eluted at approximately $1.5xV_o$ and coeluted with free ^{125}I-h_rIGF-I. This material was defined as "free". Region III included ^{125}I that eluted at V_t, i.e., coeluted with free ^{125}I. This material was defined as "degraded products". The radioactivity eluting in these three regions is presented as a percentage of the total radioactivity recovered from the column. The recovery of total radioactivity applied to these columns averaged 78%.

In a second experiment, pooled sera (37.5 ul) was incubated with 3 ml of either 3.5 M acetic acid or buffer A for two hours at room temperature. The samples were concentrated and redissolved as described above. Then ^{125}I-h_rIGF-I ($1.5x10^5$ cpm; 100 ul) was added to each sample and incubated for 16 h at 4^oC. These samples were fractionated on Sephadex G-50 as above. The recovery of total radioactivity applied to these columns averaged 74%.

Partially purified carrier proteins. Two ml of pooled sera was acidified with 120 ul of glacial acetic acid and incubated for 16 h at 4^oC. The sample was applied to a column (2.5x40cm) containing Sephadex G-50 (fine) that had been equilibrated in 1.0 M acetic

acid. The sample was eluted in an ascending manner at a flow rate of 27 ml/h with 1.0 M acetic acid. The eluate was monitored for absorbance at 280 nm and 3 ml fractions were collected. Fractions which eluted at void volume and had significant absorbance were pooled, lyophilized, dissolved in 10 ml of deionized water, and stored at -20°C until use. Once thawed, samples were diluted 1:8 and 1:16 (final dilutions 1:40 and 1:80, respectively) in RIA buffer. To test direct effects of the carrier proteins in the RIA, 50 ul aliquots of these diluted samples were added to known amounts of h_rIGF-I in a final volume of 400 ul RIA buffer containing first antibody (1:8000 final dilution). Samples were subsequently processed as described for the IGF-I RIA.

Gel filtration chromatography to separate the carrier proteins from IGF-I. For these experiments, pooled sera was labeled with ^{125}I-h_rIGF-I of varied specific activity. For this purpose, h_rIGF-I was dissolved at concentrations of 0, 25, 50, 150, and 250 ng/ml in buffer C. 200 ul of each standard solution was added to ^{125}I-h_rIGF-I (200 ul; 2.4×10^5 cpm) and mixed. After incubation for 10 minutes at room temperature, pooled sera (50 ul) were added and samples were incubated for 16 h at 4°C. One-half of each sample was added to 2 ml of 3.5 M acetic acid while the other half was added to 2 ml of Buffer A. After incubation for 2 h at room temperature, all samples were frozen and stored at -20°C. Upon

thawing, blue dextran (1 mg in 100 ul buffer D) and sucrose (0.47 M) were added to 1 ml aliquots. Acetic acid treated samples were applied to a column (1.5x30cm) containing Sephadex G-50 fine that had been equilibrated in 0.5 M acetic acid, 0.02% (w/v) sodium azide, pH 1.8. Samples were eluted in equilibration buffer in a descending direction at a flow rate of 25 ml/h. ^{125}I in each 1 ml fraction was measured and the levels of bound, free, and degraded ^{125}I were calculated. The recovery of total radioactivity applied to these columns averaged 73%.

Chromatographic analysis of samples diluted in buffer A were similar with minor modifications to those used for the acidic samples. First, 10 mg BSA, as well as blue dextran and sucrose were added to thawed samples. The BSA improved the recovery from the columns without affecting the distribution of free and bound ^{125}I-h_rIGF-I. Second, the Sephadex G-50 was equilibrated in buffer D (pH 7.4) and the samples were eluted in this buffer. Other parameters were identical and the chromatograms were evaluated using the same criteria. The recovery of total radioactivity applied to these columns averaged 76%.

Separation of IGF-I from carrier proteins by ultrafiltration. Ultrafiltration membranes with a 30,000 molecular weight cutoff (Centricon-30) were purchased from Amicon Corp. (Danvers, MA). The membranes were pretreated with 3.5 M acetic acid by percolating 5-7

ml of acid through the ultrafiltration devices over a three day period. Next, the membranes were extensively rinsed with deionized water and soaked overnight in a solution containing 10 mM Tris-HCl, 0.5% (w/v) BSA and 0.01% (w/v) thimersol, pH 7.4. Finally, the devices were thoroughly rinsed with deionized water and stored in 0.01% (w/v) thimersol. Before use, ultrafiltration devices were rinsed with deionized water and shaken to remove excess water.

The procedure which was validated for separating IGF-I from its carrier proteins using ultrafiltration is described below. Serum (75 ul) was added to 6 ml of 3.5 M acetic acid and incubated for 2 h at room temperature. Aliquots (2 ml) were transferred to Centricon-30 ultrafiltration devices. Each sample was filtered in duplicate. After centrifugation at 6000 rpm for 75 minutes at $4^{o}C$ (Sorvall SS-34 or SM-24 rotors give identical results), the ultrafiltrate was transferred to a 12x75mm polypropylene test tube. The membranes were washed an additional two times with 2 ml of 3.5 M acetic acid and ultrafiltrates were pooled. Pooled eluates were concentrated in the Speed Vac^{R} with heat lamp setting at 50%. After the final wash, the ultrafiltrate cups were rinsed with one ml of 3.5 M acetic acid which was transferred to the appropriate tube. The final concentration step was performed to dryness overnight with the heat lamp and sample chamber heater off. Samples were then dissolved in 1 or 2 ml of RIA buffer and 50 ul aliquots were assayed in duplicate in the IGF-I RIA described previously. Repeated analysis of the pooled

sera by this procedure gave a value of 213 ±8 ng/ml (mean ±SD averaged over 5 assays) with a interassay coefficient of variation of 13%. The intraassay coefficient of variation was 8.6%.

In validating this procedure, an initial experiment was performed that paralleled the studies using Sephadex G-50 chromatography. For this purpose, pooled sera were labeled in a similar manner with ^{125}I-h_rIGF-I that varied in specific activity. Various quantities of h_rIGF-I (0, 25, 50, 150, and 250 ng/ml buffer C; 300 ul) were incubated with ^{125}I-h_rIGF-I ($3.6x10^5$ cpm; 300 ul) for 10 minutes at room temperature. Pooled sera (75 ul) were added to each mixture and incubated for 16 h at 4°C. Six ml of 3.5 M acetic acid was added and the radioactivity in 1 ml aliquots was measured. The samples were processed by the ultrafiltration technique described above except that the ultrafiltrates were not concentrated immediately. The original ultrafiltrates plus the washes were combined in a single tube prior to measurement of ^{125}I. The recovery of radioactivity in the ultrafiltrate as a percentage of the radioactivity added to the ultrafiltration devices was calculated. To determine the effect of pH, the above experiment was repeated at neutral pH. To do so, buffer A was substituted for 3.5 M acetic acid in the above procedure. In addition, the amount of radioactivity retained by the membranes was measured to assess total recovery. The total recovery averaged between 85 and 95% for both neutral and

acidic conditions.

The validity of the ultrafiltration method was also assessed by the method of additions using unlabeled h_rIGF-I. Known amounts of h_rIGF-I (0, 25, 50, 150, and 250 ng/ml buffer C; 300 ul) were incubated with 75 ul pooled sera for 16 h at 4°C. Samples were processed by the ultrafiltration technique and analyzed for IGF-I content in the RIA.

Finally, the ability of the ultrafiltration method to detect differences between samples previously known to differ greatly in pGH concentration (and putatively in IGF-I concentration) was tested. Sera were collected from swine chronically treated with pGH; a treatment known to increase IGF-I levels (47,83). Yorkshire-Duroc barrows (50 kg body weight) were treated for 35 days with 70 ug pGH·kg $BWt^{-1}\cdot d^{-1}$ by im injection. Sera from these animals were kindly provided by Dr. T.D. Etherton, The Pennsylvania State University. The details of this study have been submitted for publication (2). In addition, a serum sample obtained from a HYPOX pig was kindly provided by Dr. R. Kraeling, USDA, Richard Russell Research Lab and assayed. These samples were also assayed after acidification and concentration (i.e., without the filtration step of the ultrafiltration procedure). To do this, samples were diluted in acetic acid, incubated for 2 h at room temperature, and then concentrated to dryness. Samples were subsequently dissolved in RIA buffer and assayed for IGF-I as described previously.

RESULTS

When pooled sera were assayed for IGF-I content by RIA, pretreatment conditions markedly affected the results (table 1). IGF-I values increased in samples treated to release IGF-I from carrier proteins compared to untreated sera. Pretreatment with heparin resulted in the largest increase (18-fold) and acid-ethanol treatment generated the smallest increase (9-fold) above untreated sera. In addition to these variable sera estimates, the age of animals from which sera was prepared influenced results (data not shown). Dilutions of adult sera that had been treated with acid ethanol gave displacement curves in the RIA that paralleled curves generated with h_rIGF-I. Displacement curves produced with neonatal sera treated with acid ethanol were not parallel to curves obtained with recombinant IGF-I. Also, heparin pretreatment proved to be inappropriate for neonatal samples because the interassay coefficient of variation exceeded 50% for many samples.

Together, these variable results suggested that the presence of specific carrier proteins for IGF-I in the reaction mixture might be interfering with the antibody/IGF-I reaction. Two approaches were taken to evaluate this problem. First, the effects that these various treatments had on the ability of serum carrier proteins to bind ^{125}I-h_rIGF-I was investigated. Second, the effects of the carrier proteins in the IGF-I RIA were directly tested by performing the RIA on samples containing known amounts of h_rIGF-I in the

TABLE 1. IGF-I LEVELS IN POOLED SERA ASSAYED DIRECTLY AND FOLLOWING TREATMENTS WHICH DISSOCIATE IGF-I FROM CARRIER PROTEINS

Treatment[1]	IGF-I ng/ml[2]
None	8 ±1[a]
Glycylglycine-HCl	117 ±7[b]
Heparin	147 ±6[c]
Acid Ethanol	77 ±11[d]

[1]The IGF-I values were measured in a single assay. Similar relative values between treatments were observed in subsequent assays.

[2]Values are given as mean ±SD for 4 dilutions assayed in duplicate in the RIA.

[a,b,c,d]Values with different superscripts are different ($p<0.001$) by Student's two-tailed t-test.

presence and absence of partially purified carrier proteins.

When ^{125}I-h_rIGF-I was added to pooled sera and samples were chromatographed on a Sephacryl S-200 column at neutral pH, approximately 75% of the recovered radioactivity eluted in the high molecular weight region (K_d= 0.25-0.45, figure 3). The remaining radioactivity eluted at a volume consistent with free ^{125}I-h_rIGF-I (K_d= 0.60-0.80). Thus, the majority of added ^{125}I-h_rIGF-I appeared to be complexed with carrier proteins. Incubation of radiolabeled sera with heparin for 48 h at 37°C facilitated the dissociation of hormone from these binding sites. As shown in figure 4, the ratio of bound to free ^{125}I-h_rIGF-I in the heparin treated sample was 1:2. An elution profile identical to figure 2 was obtained when labeled serum was incubated in the absence of heparin prior to chromatography (data not shown). The results of a series of chromatographic studies evaluating the effects of various treatments on the relative amounts of complexed and free ^{125}I-h_rIGF-I are summarized in table 2. The effectiveness of treatments for dissociating labeled hormone from these high molecular weight binding sites was glycylglycine-HCl at acid pH > heparin > acid ethanol. No dissociation occurred when the glycylglycine-HCl incubations were performed at neutral pH indicating that this process requires an acidic environment. Finally, none of the tested methods resulted in complete dissociation of ^{125}I-h_rIGF-I from binding sites in sera.

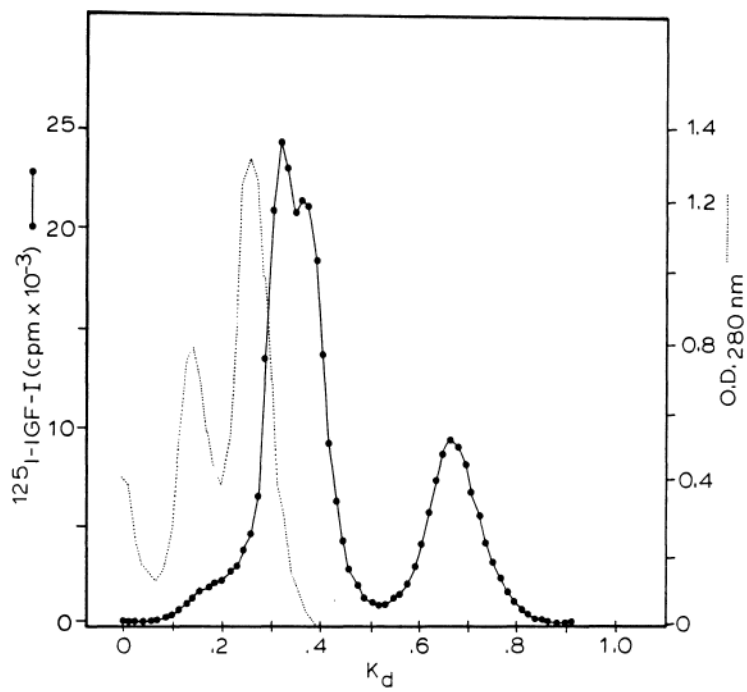

Figure 3. Elution profile of pooled sera labeled with $^{125}h_rIGF-I$ on Sephacryl S-200. Pooled sera containing $^{125}I-h_rIGF-I$ was chromatographed at neutral pH as described in "MATERIALS AND METHODS".

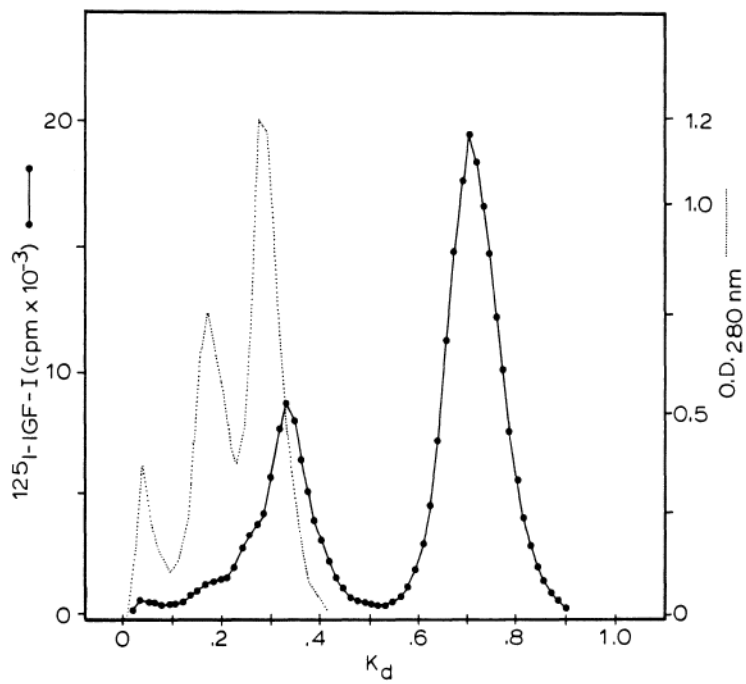

Figure 4. Effect of heparin treatment on the elution profile of pooled sera containing ^{125}I-h_rIGF-I on Sephacryl S-200. Sample was treated with heparin (40 units) and chromatographed at neutral pH as described in "MATERIALS AND METHODS".

TABLE 2. THE EFFECT OF VARIOUS TREATMENTS ON THE DISTRIBUTION OF ^{125}I-IGF-I BETWEEN BOUND AND FREE STATES AS MEASURED BY GEL FILTRATION CHROMATOGRAPHY

Treatment[1]	% Low[2] Molecular Weight	% High[2] Molecular Weight
Control[3]	29	71
Acid Ethanol	42	58
Glycylglycine-HCl, pH<3.6	86	14
Glycylglycine-HCl, pH=7.0	27	73
Heparin	67	33

[1]See figure 1 for a description of the methods used for these treatments.

[2]The radioactivity eluting in these two regions was summed and the relative distribution in each peak was calculated.

[3]The control sample was incubated for 48 h at 37°C prior to chromatography.

In the initial series of studies discussed above, larger volumes of serum were used than those analyzed in the IGF-I RIA. In the remaining experiments, 1:80 dilutions of sera were used to more closely simulate the conditions employed in the RIA. When compared to the chromatographic results obtained with higher volumes of sera, a 1:80 dilution of sera resulted in a 3-4-fold decrease in the relative percentage of ^{125}I that eluted in the bound region (figure 5). The bound radioactivity now accounted for only 20% of the recovered radioactivity. A similar dilution of labeled sera in 3.5 M acetic acid and fractionation of samples on Sephadex G-50 in the presence of acetic acid resulted in a dissociation of >95% of hormone from carrier proteins (figure 5).

Some investigators have argued that acidification of human sera denatures the carrier proteins and alters their affinity such that they do not interfere in the antigen-antibody reaction when the sample is subsequently neutralized (82,86). Therefore, the effect of acidification of swine sera on the affinity of carrier proteins for IGF-I was investigated. Pooled sera were labeled with ^{125}I-h_rIGF-I, acidified with acetic acid, and then concentrated to dryness. When this sample was dissolved in neutral buffer and chromatographed on Sephadex G-50 equilibrated with neutral buffer, the elution profile was identical to that of pooled sera that had not been acidified (figure 5). Samples of labeled sera that were prepared and chromatographed in neutral buffers, yielded similar

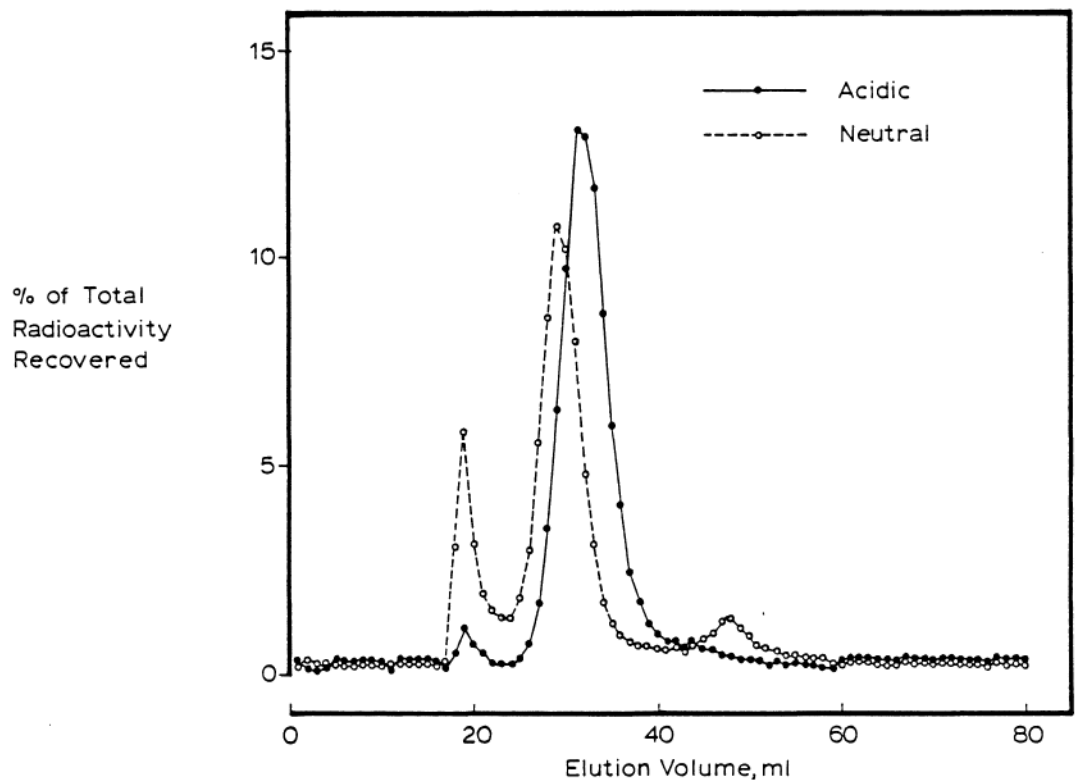

Figure 5. Effect of acetic acid on the elution profile of labeled sera fractionated by Sephadex G-50 gel filtration chromatography. Samples of pooled sera labeled with ^{125}I-h_rIGF-I were diluted 1:80 in either acetic acid (pH 1.8) or neutral Tris-HCl buffer (pH 7.4). These samples were subsequently fractionated on columns of Sephadex G-50 equilibrated in their respective diluent as described in "MATERIALS AND METHODS". The recovery of radioactivity from the acidic and neutral columns was 75% and 72%, respectively.

results. Similarly, when radiolabeled samples that had been initially acidified were subsequently fractionated on Sephadex G-50 with neutral buffer, approximately 20% of ^{125}I-h_rIGF-I eluted with high molecular weight material. Together, these results indicate that acidification of swine sera does not irreversibly decrease the affinity of the carrier proteins for IGF-I.

The direct effect of partially purified carrier proteins on the IGF-I RIA was investigated next. Levels of carrier proteins equivalent to those present in 1:40 and 1:80 dilutions of serum were added to tubes containing various amounts of h_rIGF-I (10-640 pg). At low concentrations of h_rIGF-I, there was significantly higher displacement of ^{125}I-h_rIGF-I from the antibody in the presence of the carrier proteins than in their absence (figure 6). Furthermore, when carrier proteins were present, the amount of ^{125}I bound to the antibody did not change when the total quantity of IGF-I was 10-160 pg. ^{125}I was displaced from the antibody when the amount of h_rIGF-I was 320 and 640 pg at 1:80 and 1:40 dilutions of carrier proteins, respectively. These results demonstrate that the carrier proteins effectively compete with the antibody for binding of labeled IGF-I.

The finding that the carrier proteins interfered in the antibody-antigen reaction mandated development of a method to separate IGF-I from these proteins prior to the RIA. One approach that has been successfully utilized involves gel filtration chromatography under

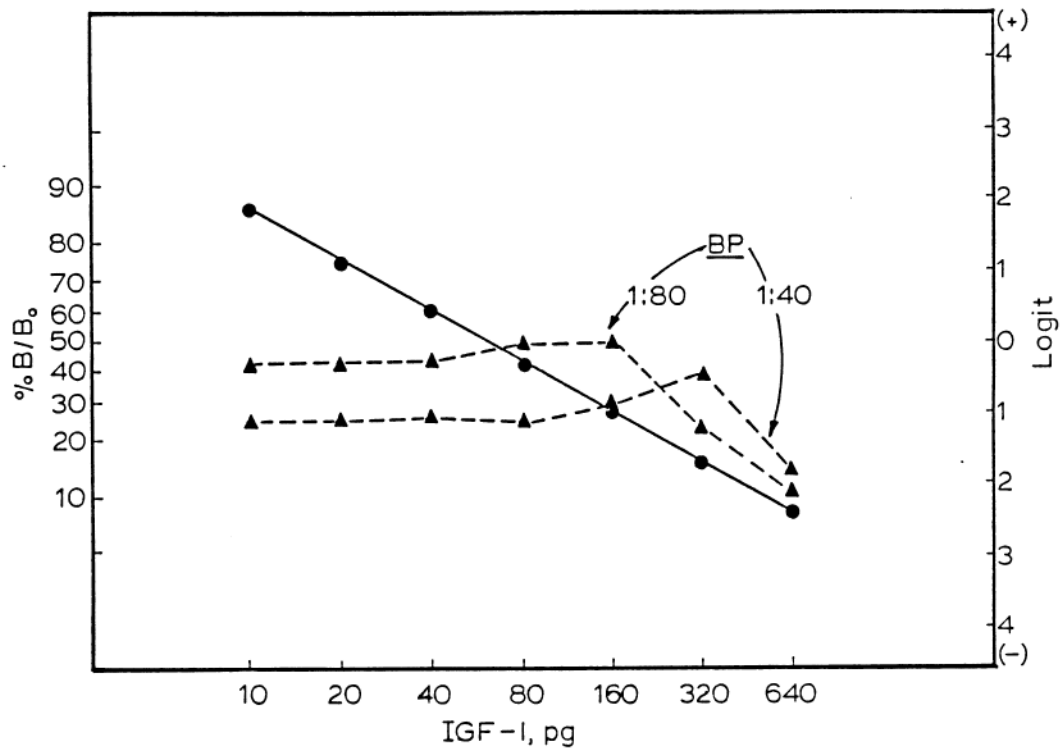

Figure 6. Effect of direct addition of carrier proteins on the IGF-I RIA antigen-antibody reaction. Carrier proteins were partially purified as detailed in "MATERIALS AND METHODS". The IGF-I RIA was performed on samples containing the indicated quantities of h_rIGF-I in absence (solid line) and presence (dashed lines) of carrier proteins (BP).

acidic conditions (36). Due to some inherent disadvantages (see discussion) with this technique, the feasibility of using ultrafiltration was investigated as an alternative to low pressure gel filtration chromatography. An initial experiment was performed to compare the recovery of ^{125}I-h_rIGF-I from serum using ultrafiltration vs. gel filtration chromatography. For this experiment, pooled sera were labeled with ^{125}I-h_rIGF-I. Identical aliquots were diluted in either acetic acid or neutral Tris-HCl buffer. Then the samples were either chromatographed on Sephadex G-50 equilibrated in their respective diluents or processed by the ultrafiltration procedure in their respective diluents. Results from chromatographic analyses are presented as the percentage of recovered radioactivity that eluted as bound, free, or degraded material (figure 7). When maintained in acetic acid, greater than 91% of the recovered radioactivity eluted as free hormone (figure 7A). The amounts of radioactivity eluting as bound and degraded material were less than 5%. In addition, the distribution of radioactivity was not influenced by the specific activity of the ^{125}I-h_rIGF-I. In contrast, when neutral conditions were used for sample pretreatment and chromatographic analysis, only 70-80% of the recovered radioactivity eluted as free hormone (figure 7B). Approximately 10-20% of the ^{125}I eluted as complexed hormone, while less than 10% of the radioactivity eluted in a position corresponding to degradation products. The distribution of ^{125}I was also

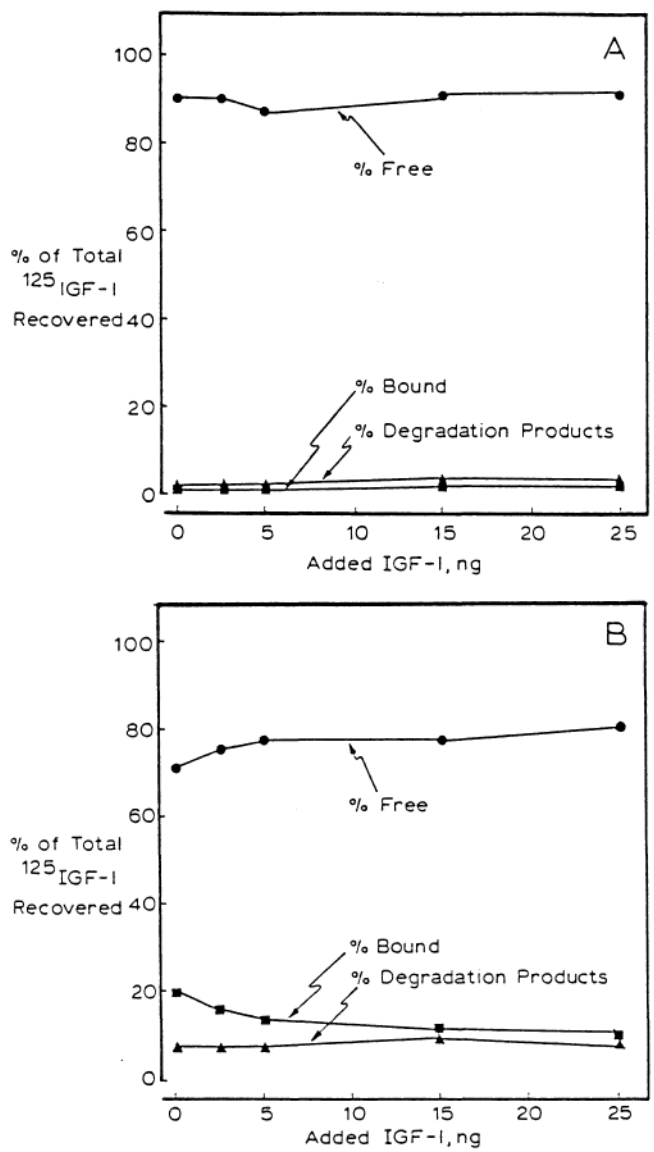

Figure 7. Chromatographic analysis of labeled sera under neutral and acidic conditions and in the presence of varying amounts of unlabeled h_rIGF-I. Aliquots of ^{125}I-h_rIGF-I were spiked with h_rIGF-I (0, 2.5, 5, 15, and 25 ng) and added to pooled sera as described in "MATERIALS AND METHODS". A) The labeled serum was treated with acetic acid (pH 1.8) followed by chromatography on Sephadex G-50 equilibrated in acetic acid; B) The labeled serum was treated with neutral Tris-HCl buffer (pH 7.4) followed by chromatography on Sephadex G-50 equilibrated in neutral Tris-HCl buffer.

relatively independent of the level of h_rIGF-I added to pooled sera.

Data from the evaluation of separation by ultrafiltration are presented as the percentage of total radioactivity recovered in the ultrafiltrate. Results from the ultrafiltration studies were similar to those obtained with gel filtration chromatography. When acidic conditions were used for sample pretreatment and for ultrafiltation, approximately 63% of the added radioactivity was recovered in the ultrafiltrate (figure 8). This percentage was largely unaffected by the specific activity of the tracer. The percentage of radioactivity present in the ultrafiltrate when samples were pretreated and ultrafiltered in 50 mM Tris-HCl at neutral pH was markedly influenced by the specific activity of the ^{125}I-h_rIGF-I (figure 8). Only 25% of the total radioactivity added was present in the ultrafiltrate when less than 5 ng of unlabeled hormone were present. When greater than 15 ng of h_rIGF-I was present, approximately 50% the total ^{125}I added was recovered in the ultrafiltrate.

The apparent molecular weight of the radiolabeled material in the ultrafiltrate was determined by Sephadex G-50 gel filtration chromatography. About 90% of the radioactivity eluted as free ^{125}I-h_rIGF-I (data not shown). The remainder of the radioactivity eluted as degradation products, while minimal amounts eluted as bound hormone.

The validity of separation of carrier proteins from IGF-I by the

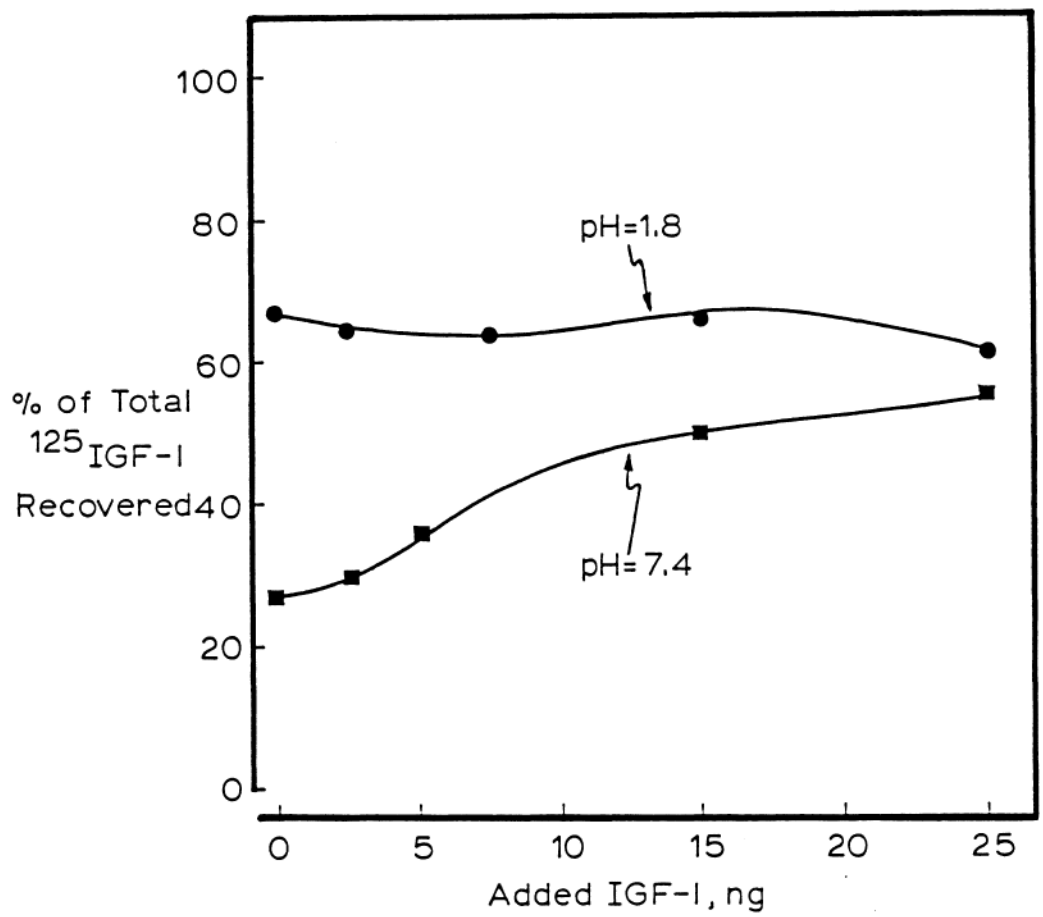

Figure 8. Effect of pH and specific activity on the recovery of ^{125}I-h$_r$IGF-I from pooled sera by the ultrafiltration procedure. Samples were prepared identically as described in legend to figure 7 and processed by the ultrafiltration procedure (see "MATERIALS AND METHODS") at pH 1.8 or 7.4. The results are given as a percentage of total radioactivity recovered in the ultrafiltrates.

ultrafiltration procedure was also assessed by the method of additions. Various concentrations of h_rIGF-I were added to pooled sera. After incubation overnight, the samples were processed by the ultrafiltration method. h_rIGF-I, added to pooled sera, produced a dose dependent increase in the measured IGF-I that was linear (R^2=0.98) over the concentration range 100-1000 ng/ml (figure 9). The recovery averaged 72% of the added material in two experiments.

In a final validation step, the ultrafiltration method was used to determine IGF-I levels in sera from pigs treated with pGH for 35 consecutive days. In this experiment, the necessity of filtration was also tested by leaving this step out of the ultrafiltration procedure. When these samples were acidified and then concentrated to dryness without being filtered, markedly lower values were measured than when the filtration step was included (table 3). Serum values of IGF-I in samples from control animals and those treated with pGH were 7- and 9-fold, respectively, lower than those measured for ultrafiltrates from these groups. Serum IGF-I of pGH treated animals was 2.6-fold higher than control levels when the filtration step was included in sample processing. When the filtration step was omitted, the measured level of IGF-I in the pigs treated with pGH was only 2-fold higher than control levels. The serum value for the HYPOX animal was not affected by omission of the filtration step. Consequently, the HYPOX animal had IGF-I levels that were 24-fold less than controls when filtration was included and only a 3.2 fold decrease when this step was omitted.

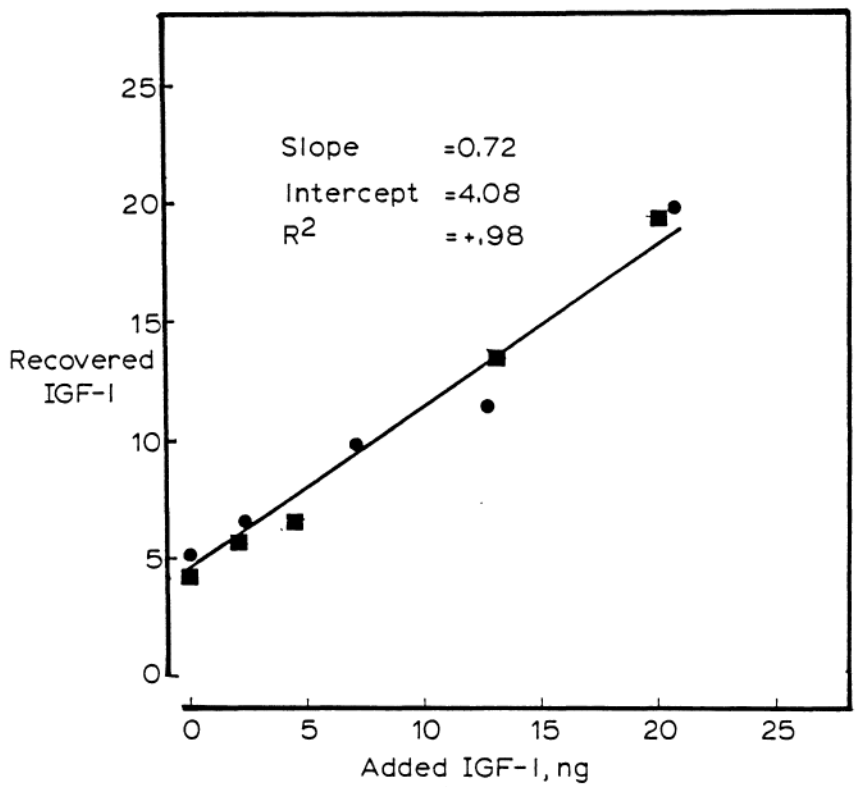

Figure 9. Recovery of unlabeled IGF-I from pooled sera by the ultrafiltration procedure. h_rIGF-I was added to serum at the indicated dose levels on the figure and incubated 16 h at 4°C. The serum was processed by the ultrafiltration method and analyzed for IGF-I content in the RIA. The results of two similar experiments are shown.

TABLE 3. COMPARISON OF IGF-I LEVELS IN SERA FROM CONTROL, pGH TREATED, AND HYPOX SWINE AS MEASURED BY ACIDIFICATION/CONCENTRATION (A) AND ULTRAFILTRATION (A+F)[1].

Sample	N	Serum IGF-I	
		A	A+F
		ng/ml	
Control	8	39 ±4[a]	268 ±21[b]
pGH treated	8	80 ±4[c]	693 ±25[d]
HYPOX	1	12 ±1[2]	11 ±1[2]

[1]All values were determined in the same assay. The values for column A were determined by omitting the filtration step in the ultrafiltration procedure described in "MATERIALS AND METHODS".

[2]Values are given as mean ±SD of triplicate samples assayed in duplicate in the RIA.

[a,b,c,d]Values are given as mean ±SEM Different superscripts denote differences at $p<0.001$ level.

DISCUSSION

Most RIAs for IGF-I incorporate a step which dissociates IGF-I from its carrier proteins before the assay (36,60,61,78,81). When the antisera utilized in this study was initially characterized (38), a nonequilibrium technique was employed for the measurement of IGF-I in untreated human sera. Although this technique accurately reflects the GH dependency of IGF-I, it was subsequently found to underestimate serum IGF-I values (60). This conclusion was based on the finding that incubation of sera at an acid pH increased the amount of detectable IGF-I. When this antisera was used to measure IGF-I in other species (41,48,80), the GH dependence of IGF-I was not evident unless sera were first pretreated with acid. It has been suggested that the carrier proteins from these species have a higher affinity for IGF-I than human carrier proteins and therefore, compete with the human anti-IGF-I antibodies for IGF-I. Furthermore, it is believed that acidification of sera decreased the affinity of the carrier proteins for IGF-I. This alteration negated interference of carrier proteins in subsequent assessment of IGF-I by RIA.

The low values that were measured in untreated swine sera compared to those in treated samples (table 1) indicated that swine carrier proteins have an affinity for IGF-I of sufficient magnitude to compete with the antibody for IGF-I. However, the variable results obtained with different types of treatment protocols (heparin > glycylglycine-HCl > acid ethanol) suggested that swine carrier

proteins may still be capable of competing with the antibody for IGF-I following such treatments.

Chatelain et al. (60,61) have examined the effects of neutral incubation of sera and heparin on the carrier protein-IGF-I complex in human sera. They observed that incubation of sera (24 h, 37°C) at neutral pH resulted in a 140% increase in immunoreactive (IR)-IGF-I (60). Since this process was dependent on the length and temperature of incubation and was blocked by several protease inhibitors, these investigators suggested that proteolysis was involved in the mechanism responsible for these increments. When incubated sera samples were chromatographed on Sephacryl S-200 at neutral pH and compared to similar chromatography of non-incubated samples, increments of IR-IGF-I were observed in almost every fraction (60). However, an increase in free IGF-I was not apparent and therefore, could not account for the increments in IR-IGF-I measured in sera that had not been chromatographed. The authors proposed that the conformation of the IGF-I-carrier protein complex was altered (without dissociation) by a proteolytic event thereby facilitating binding of IGF-I by antibody.

When Clemmons et al. (61) incubated heparinized plasma for 24 h at 37°C, about 80% of the IR-IGF-I exhibited an apparent molecular weight of <20 kDa by gel filtration chromatography. This result implied that heparin effectively dissociated IGF-I from carrier proteins. Furthermore, since heparin(s) is extremely anionic (87),

it may compete directly with carrier proteins for complexation of IGF-I. If so, the presence of heparin may itself produce artifactual results in the RIA.

Bala and Bhaumick (88) have also investigated the effects of heparin in the RIA for IGF-I. In their protocol, human sera were acidified with formic acid, lyophilized, and diluted in neutral buffer prior to RIA (78). The presence of heparin in the sample increased the level of IR-IGF-I by 100% compared to sera assayed in the absence of heparin. This difference was eliminated by chromatography of heparinized plasma on Sephadex G-75 equilibrated in 1% formic acid (pH 2.3) prior to RIA (88). They also found that the presence of heparin in RIA buffer did not displace labeled IGF-I from the antibody (88). Consequently, they suggested that heparin increased the apparent level of IR-IGF-I in sera by a process that required additional factors in serum.

Incubation of labeled porcine sera with heparin at neutral pH prior to fractionation by gel filtration chromatography resulted in the partial release of labeled hormone from high molecular weight binding sites (figure 3, table 2). These results were similar to Clemmons et al. (61); however, their elution buffer contained heparin and this may have prevented reassociation of IGF-I and carrier proteins. Also, addition of heparin to RIA buffer failed to displace labeled antigen from the antibody (data not shown). This result is consistent with the observation of Bala and Bhaumick (88). Taken

together, the results discussed above would indicate that heparin treatment of swine sera is an unreliable method for assessing IGF-I.

The effectiveness of treatment of sera with glycylglycine-HCl (glygly) at pH 3.6 and acid ethanol in facilitating dissociation of IGF-I from carrier proteins was also investigated. Such dissociation is probably independent of proteolytic activity, but instead, is more likely due to a direct disruption of the noncovalent interactions between IGF-I and carrier proteins. Chatelain et al. (60) have demonstrated that the increase in IR-IGF-I measured after treatment of human sera with glygly was not inhibited by elevated temperatures (20-50^{o}C), occurred at a fast rate (1 h) compared to heparin treatment (24-48 h), and was not blocked by protease inhibitors. Furthermore, the acid induced increment in IR-IGF-I had a narrow pH optimum (3.6-4.0), suggesting participation of carboxyl groups in the interaction between IGF-I and carrier proteins. Although the authors suggested that acid proteases were involved in the dissociation process, the presence of acid proteases in serum is unlikely unless cell damage had occurred.

When porcine sera labeled with ^{125}I-h_rIGF-I was fractionated on Sephacryl S-200 at neutral pH (figure 3, table 2), a majority (75%) of the radioactivity eluted in the high molecular weight region. The elution volume of this material indicated an apparent molecular weight of 20-45 kDa. Furthermore, labeled h_rIGF-I was displaced from these sites when samples were incubated in glygly

buffer at acidic, but not neutral, pH (table 2). These results indicate that the major binding species in swine sera has a similar molecular weight to the small molecular weight carrier protein found in the sera of other species (46-48,55,80,83). In addition, acid lability of this complex is similar to that found in other species (36,41,48,60,80,83). However, when labeled swine sera were extracted with acid ethanol (figure 1) and chromatographed on Sephacryl S-200 at neutral pH, 58% of the radioactivity eluted in the high molecular weight region (table 2). One difference between these two procedures that probably accounts for this discrepancy is that glygly treated sera were directly chromatographed under neutral conditions, whereas acid ethanol treated sera were neutralized and lyophilized before similar fractionation under neutral conditions. Thus, the carrier proteins may not have been irreversibly denatured and reassociation with IGF-I occurred. This possibility is supported by the observation that when labeled porcine sera were incubated in acetic acid (pH 1.8) and neutralized before and during gel filtration chromatography approximately 20-25% of the recovered radioactivity eluted as complex (data not shown). This percentage is equivalent to the amount of complex that eluted when labeled porcine sera that had never been acidified were similarly chromatographed (figure 5). In contrast, maintenance of labeled swine sera at an acidic pH during all steps resulted in complete dissociation of IGF-I from carrier proteins (figure 5).

Similar results have been obtained with human sera by Bala and coworkers (78,86). Using the acidification and lyophilization procedure described above, they found that the carrier proteins effectively bound ^{125}I-IGF-I when analyzed by gel filtration chromatography at neutral pH. However, the procedure eliminated the interference by the carrier proteins in the RIA. Thus, they concluded that acidification altered the affinity of the carrier proteins for IGF-I, thereby facilitating quantitative binding of IGF-I by the antibody. In contrast, swine carrier proteins isolated by gel filtration chromatography in 1 M acetic acid interfered in the RIA (figure 6). Two explanations for these results are possible. Either the affinity of swine carrier proteins for IGF-I is not decreased by exposure to acid or the magnitude of the change in affinity for IGF-I is not sufficient to eliminate competition between swine carrier proteins and the human anti-IGF-I for the hormone.

Zapf et al. (36) have also investigated effects of the carrier proteins in their RIA. These investigators used Sephadex G-50 gel filtration chromatography in 1M acetic acid to separate IGF-I from carrier proteins before RIA. In one experiment, they separately pooled and lyophilized the carrier protein fractions and the IGF-I frations from a number of serum samples. The IGF-I pool was then assayed in the absence and in the presence of carrier proteins. Measured levels of IGF-I in sera from normal patients were consistently lower in the presence of the carrier proteins than in

their absence. In contrast, carrier proteins isolated from sera of acromegalic patients yielded the opposite results. These data contradict those of Bala and coworkers (86) who found that simple acidification of human sera eliminated interference by carrier proteins in their RIA. Since factors which control the amount of carrier proteins in serum are currently not well understood, Zapf et al. (36) concluded that removal of the carrier proteins is essential before measuring IGF-I levels by RIA.

The conclusion reached from the above results is that a method for measuring IGF-I in swine sera by RIA requires both dissociation and subsequent separation of IGF-I from carrier proteins. One method already discussed for separating IGF-I from its carrier protein is gel filtration chromatography in 1 M acetic acid (36). This method has several disadvantages. These include the length of time required to calibrate columns and fractionate samples. Consequently, a relatively small number of samples can be processed in a day (10-20 with five columns). Also, there are marked nonspecific losses because small amounts of material are applied to relatively large volumes of resins. Finally, elution profiles are variable due to "drifts" in elution peaks with column aging. This complicates interpretation of results. Another method that has been used for separating IGF-I from carrier proteins in culture medium conditioned by hepatocytes is high performance liquid chromatography (HPLC; 68). In addition to the problems that might be encountered in adapting

this method to serum, HPLC requires expensive equipment and technical training. Because of these problems, ultrafiltration using Amicon 30 kDa molecular weight cutoff membranes was investigated as an alternative procedure for separation of IGF-I and carrier proteins. This technique has several advantages compared to gel filtration chromatography and HPLC. First, calibration of membranes is minimal. Second, larger numbers of samples can be run in a day (48 using two SM-24 rotors). Third, the surface area of the membrane is smaller than the surface area of chromatographic beds, thereby reducing nonspecific losses. Finally, ultrafiltration is a simple procedure that requires little technical training.

To determine the feasibility of ultrafiltration as a means of separating IGF-I from carrier proteins, this technique was compared with Sephadex G-50 gel filtration chromatography. Porcine sera were labeled with ^{125}I-h_rIGF-I that varied in specific activity and then subjected to either gel filtration chromatography or ultrafiltration. For comparison, both techniques were conducted under acidic and neutral conditions. The percentage of radioactivity recovered either in the eluate as "free" IGF-I from columns or that in the ultrafiltrate was chosen as the basis for comparison. As expected, when neutral conditions were employed for gel filtration chromatography and ultrafiltration, recoveries were low (figures 7B and 8). Furthermore, recovery was affected by the specific activity of the radiolabel in both assays, although the magnitude of the

change was minimal in the chromatographic technique. These results suggest that the affinity of IGF-I for carrier proteins is great at neutral pH.

When sera were applied to columns containing Sephadex G-50 equilibrated with acetic acid, the recovery of ^{125}I-h_rIGF-I was similar to values reported by Zapf et al. (36). These investigators recovered 63% of the applied radioactivity in the peak corresponding to free IGF-I when labeled human serum was chromatographed on Sephadex G-50 equilibrated in 1 M acetic acid. The ^{125}I-IGF-I preparation they used was only 81% precipitable with 10% TCA. Assuming that the radioactivity eluting in this free peak was 100% precipitable by 10% TCA, they calculated a recovery of 78% of the TCA precipitable radioactivity. In the present study, an average of 91% of the recovered radioactivity eluted as free hormone under identical conditions (figure 7A). The average recovery of total radioactivity added to these columns was 73%; which means, about 66% of the input radioactivity was recovered in the free IGF-I peak. The ^{125}I-h_rIGF-I tracer used in this study was about 90% precipitable with 5% TCA. Therefore, about 74% of the TCA precipitable radioactivity was recovered in the free peak. This is in close agreement with the 78% recovery reported by Zapf et al. (36).

The recovery of ^{125}I-h_rIGF-I from porcine sera using ultrafiltration under acidic conditions was 64%. When the ultrafiltrates were analyzed by gel filtration chromatography at

neutral pH, approximately 91% of the radioactivity eluted as free IGF-I. The remainder eluted as degradation products. Thus, about 58% of the applied labeled hormone was recovered in the ultrafiltrate as "free" IGF-I. This level is equivalent to a recovery of TCA precipitable radioactivity of 64% which was 87% of that recovered by acidic gel filtration chromatography. Despite the lower recovery, minimal radioactivity eluted as bound hormone when the ultrafiltrates were chromatographed at neutral pH. This finding indicates that the carrier proteins were quantitatively excluded by the membrane, since IGF-I reassociated with carrier proteins after acidification and subsequent fractionation on neutral gel filtration columns (figure 5). Moreover, it indicates that the apparent molecular weight of the carrier protein exceeded 30 kDa.

The effect of ultrafiltration on the RIA was further characterized by the method of additions. When h_rIGF-I was added to porcine sera, a dose dependent increase in IR-IGF-I was measured that was linear over the concentration range of 150-1000 ng/ml (figure 8). The recovery averaged 72% in two separate experiments. The increase in recovery of unlabeled h_rIGF-I compared to the recovery of labeled peptide is perplexing. One explanation is greater affinity of ^{125}I labeled peptide for membranes than the unlabeled peptide. Zapf et al. (36) found the opposite effect viz., they obtained a lower recovery (66%) of unlabeled IGF-I compared to the recovery of labeled IGF-I by gel filtration chromatography at

acid pH.

One of the most important validation criterion for an IGF-I RIA is that it must reflect the GH dependence of IGF-I (75). In humans, this is done by showing that IGF-I levels are high in sera from acromegalics and low in sera from hypopituitary patients compared with sera from healthy individuals. The validity of an IGF-I RIA can also be confirmed by measuring increases in serum IGF-I following treatment with GH (47,83). Swine chronically treated with pGH (70 $ug \cdot kg\ BWt^{-1} \cdot d^{-1}$) had 2.6-fold higher IGF-I levels in their sera than control animals when the samples were assayed by the ultrafiltration procedure. In addition, serum from a HYPOX pig showed a 24-fold decrease in IGF-I levels compared to control animals. Thus, the ultrafiltration method for separating IGF-I from its carrier proteins accurately reflects the GH dependence of swine IGF-I.

In addition to assaying these samples by the normal ultrafiltration procedure, samples that were not subjected to ultrafiltration were also evaluated. Markedly lower values were obtained, confirming the need for separation of IGF-I from carrier proteins before RIA. When the pooled sera was assayed by this acidification/concentration (A/C) procedure a value of 16 ng/ml was measured. This value is about 7-fold less than when this sera was assayed after glygly treatment (table 1). Several differences exist between these two procedures (A/C vs. glygly) including length of

incubation (2 vs. 48 h), temperature (25°C vs. 37°C), and pH (1.8 vs. 3.6). Whether one or more of these conditions or an unknown factor contributed to the different results requires further research.

In summary, ultrafiltration is a rapid and valid method for separating IGF-I from carrier proteins prior to the RIA based on the following results. First, minimal material capable of binding labeled h_rIGF-I was found in the ultrafiltrates as judged by gel filtration chromatography. Second, recovery of ^{125}I-h_rIGF-I from swine sera was not dependent on specific activity. Third, the recovery of h_rIGF-I from swine serum was linear (R^2=0.98) over the concentration range 150-1000 ng/ml and averaged 72%. Finally, assessment of sera from swine chronically treated with pGH contained 2.6-fold higher levels of IGF-I than control samples, whereas the levels in serum from a HYPOX animal was only 4% that of normal animals.

CHAPTER 3.

AN INVESTIGATION OF THE *IN VIVO* AND *IN VITRO* EFFECTS OF pGH ON THE NEONATAL PIG

INTRODUCTION

Administration of porcine GH (pGH) to rapidly growing 30-90 kg pigs improves growth performance (1-3,89). pGH treatment improves feed efficiency, growth rate and carcass lean mass, while decreasing carcass fat content. Due to the economic considerations associated with production, widespread adoption of this treatment by the swine industry will either require another form of administration (currently intramuscular injections are employed) or treatment at an earlier point in the growth cycle.

Several reports have indicated that the IGFs are developmentally regulated in human (36,38), rat (41,90,91), and mouse (48) sera. From these data, it has been postulated that IGF-I is involved in postnatal growth (GH dependent) and IGF-II is involved in fetal growth (GH independent). In addition to IGFs, the carrier proteins also appear to be differentially expressed during development (48,91-93). In fetal and early neonatal life, the low molecular weight (30-45 kDa) non-GH dependent form of the carrier protein is the predominant binding species in sera. In contrast, the large molecular weight (>150 kDa) GH-dependent carrier protein is the major binding species in adult sera. This shift is postulated to occur when growth becomes GH dependent (91,93). In addition to these changes in form, Draznin et al. (91) have shown that the binding capacity of neonatal rat sera for IGFs increases with age. Clearly, investigators need to consider such changes in the type and level of

carrier proteins in early neonatal life in order to design suitable protocols for assessing IGF-I in neonatal sera.

Many cell types are capable of synthesizing IGFs in vitro (62-72, 94,95). Most studies have found that carrier protein synthesis by a variety of cell types is coordinately regulated with IGF synthesis. Nissley et al. (13) have made the observation that while there are cells that synthesize a carrier protein in the absence of the synthesis of IGFs, the reverse situation has not been reported. Moreover, a carrier protein synthesized by monolayer cultures of human fibroblasts in serum-free medium modulated the binding of labeled IGF-I to the cell monolayer (95). These results indicate that carrier proteins synthesized in vitro have the potential of interfering with the interaction of IGF-I with high affinity receptors (i.e., cell surface receptors or antibodies).

Results summarized in the previous chapter demonstrated that IGF-I carrier proteins present in swine sera interfere in the measurement of IGF-I by RIA. A two step procedure for preparation of pig sera for determination of IGF-I by RIA was developed and validated. In this chapter, this procedure was utilized to measure IGF-I in sera from neonatal pigs and in medium conditioned by neonatal pig hepatocytes. The specific objectives of this research are as follows: 1) measure the levels of IGF-I in sera of neonatal swine; 2) determine the effect of pGH treatment of neonatal swine on growth rate and serum levels of pGH and IGF-I; 3) determine

whether primary cultures of neonatal hepatocytes synthesize IGF-I; and 4) determine if secretion of IGF-I by isolated hepatocytes is stimulated by treatment with pGH either *in vivo* or *in vitro*.

MATERIALS AND METHODS

Animals. Male and female crossbred 3-21 d old piglets were used in this study. All animals received an intramuscular injection of iron-dextran (50mg Fe) between 1 and 2 d of age. Piglets were fasted for 3 h prior to the isolation of liver cells. Otherwise, they were allowed free access to sow's milk throughout the study and were not given further nutritional supplements.

Porcine growth hormone treatment. pGH was obtained from the National Hormone and Pituitary Program (USDA lot B-1). The biolgical activity of this material is referenced as 3.5 IU/mg (95% confidence limits, 1.5-18.1) as measured by the rat tibia test. This material was weighed and diluted in vehicle (25 mM $NaHCO_3$, 25 mM Na_2CO_3, 154 mM NaCl, pH 9.3) every fourth day and stored refrigerated. A stock solution (250 ug/ml) was used for subcutaneous injection at a dose of 50 ug·kg $BWt^{-1}\cdot d^{-1}$. Control animals (vehicle only) were matched within a litter by age and sex with pGH treated animals. Three treatment groups were established. Piglets in group I were treated with pGH for a total of 7-8 days. Injections were initiated at 3-7 d of age and continued until 10-14 d of age. Piglets in group II were similarly injected for 7-8 consecutive days, although, treatment was not initiated until 10-14 d of age. Piglets in group III were injected with pGH for a total of 14-15 consecutive days. Treatment was initiated at 3-7 d of age and continued until

17-21 d of age. Injections were made between 0800 and 0900 each day. On the final day of treatment, piglets were injected at 0630 and blood samples were obtained by vena cava puncture two hours later. Serum was harvested after blood samples were coagulated at 4°C for 6 h and centrifuged at 1800xg for 30 min. After collection of blood, piglets were anesthetized with sodium pentobarbital (100 mg iv) and hepatocytes were isolated.

Isolation of liver cells. Liver cells were isolated on the same day from each pair of experimental and control animals essentially by the method of Caperna et al. (96) with minor modifications. For 10-14 d old piglets, the procedure required no modifications. However, for animals older than 14 d of age (approximate weight range of 4-7 kg), some changes were found to be beneficial. The volumes of all perfusion solutions were doubled. The collagenase solution was increased to 250 ml and, in some cases, 300 ml. In addition, once the cannula was secured in the thoracic vena cava and the ligature around the lower vena cava was tightened, the liver was perfused at a flow rate of 35 ml/min for 15-25 sec. After severing, the portal vein, the flow rate was increased to 70 ml/min. The collagenase solution was also perfused at 70 ml/min for 20-45 min to ensure complete breakdown of the liver.

Isolation and culture of hepatocytes. Hepatocytes were isolated from the liver cell suspension by differential centrifugation (96). The average viability exceeded 75% as measured by the exclusion of trypan blue. Cells (1.5×10^6 viable cells) were added to collagen coated plates (35 mm^2; six-well cluster plates; Costar, Cambridge, MA) containing arginine-free medium 199 supplemented with 0.15 mM L-ornithine (basal medium), 0.5 ug/ml porcine insulin and 10% (v/v) fetal bovine serum (FBS) to facilitate attachment of viable hepatocytes. Cells were incubated at 37°C in 95% air:5% CO_2 for 2 h and then washed twice with 10 mM Hepes (N-2 hydroxyethyl-piperazine-N'-2-ethane sulfonic acid) buffered saline, pH 7.4. Subsequently, cells were maintained in serum-free medium. This consisted of medium 199 supplemented with 0.2% (w/v) fatty acid free bovine serum albumin (Sigma Chemical Co., St. Louis, MO), 18 uM oleic acid, 0.05 ug/ml porcine insulin, 50 ug/ml gentamycin and 0.5% (v/v) Fungizone. Cells were washed daily with 10 mM Hepes buffered saline and fresh serum-free medium was added. Visible inspection of hepatocytes after 72 hours indicated that cells appeared morphologically intact and maintained characteristic polygonal shape.

IGF-I production by isolated hepatocytes. IGF-I production was assessed by measuring the level of IGF-I in serum-free medium conditioned between 48 and 72 h in culture. In addition, the effect of pGH on IGF-I production was tested by adding increasing amounts

of pGH to serum-free medium. After 48 h in culture, monolayers were washed twice with 10 mM Hepes buffered saline prior to the addition of fresh serum-free medium containing either 0, 50, 250 or 500 ng/ml pGH (USDA lot B-1). Conditioned medium (combined from three wells) was collected 24 h later and was centrifuged at 100xg for 10 min to remove cellular debris. Samples were frozen at -70°C until assayed for IGF-I content (described below). Monolayers were washed twice with ice cold 10 mM Hepes buffered saline. Cells from three wells were quantitatively removed with two ml of 0.2% (v/v) Triton X-100. Cellular protein was precipitated with 10% (w/v) trichloroacetic acid, pelleted by centrifugation and then redissolved in 1 N NaOH. The modified Lowry method (97) was used to quantify cell protein with bovine serum albumin as standard. Data are presented as ng IGF-I/mg cell protein/24 h.

Radioimmunoassays. pGH was measured by an homologous double antibody RIA previously described (98). IGF-I was measured in neonatal sera processed by the ultrafiltration procedure described in chapter 2. Conditioned media (CM) was assayed for IGF-I by a minor modification of the ultrafiltration procedure. CM (1.0 ml) was added to 3.5 M acetic acid (3.0 ml) and incubated for 2 h at room temperature. A two ml aliquot from each sample was subjected to the ultrafiltration assay described in chapter 2. The concentrated samples were redissolved in 250 ul of RIA buffer (see chapter 2) and assayed in

duplicate as 50 ul aliquots in the IGF-I RIA. This procedure was validated in a similar manner to the protocol used for serum. The recovery of ^{125}I-h_rIGF-I from CM averaged 86% and was not dependent on the specific activity of the labeled h_rIGF-I. The recovery of h_rIGF-I from CM averaged 62% and was linear over a concentration range of 2-10 ng/ml. The lower recovery of unlabeled h_rIGF-I appeared to be due to losses in the concentration step. When known amounts of h_rIGF-I (0.44-2.40 ng) were subjected to ultrafiltration in the absence of CM, the recovery averaged 66%. These values are substantially lower than those obtained for sera. The reason for this difference is not clear, although it may in part be due to the lower mass of standard used in the CM recovery experiments compared to the sera recovery (2.25-20.65 ng) experiments. The values presented for sera and CM were not corrected for estimated losses during sample preparation.

Chromatography of conditioned medium. Hepatocytes were cultured from a control animal as described above. After 48 h in culture, the serum-free medium was removed and the monolayers were washed twice with Hepes buffered saline. Fresh serum-free medium without bovine serum albumin was added and monolayers were incubated for 24 h. The conditioned medium was pooled from two plates, centrifuged to remove cellular debris, and frozen at -70^{o}C until use. 20 ml of

conditioned medium was dialyzed against 5 mM Tris-HCl, pH 7.4 at 4°C overnight with one buffer change (Spectrapor^{R} membrane tubing, 1000 MW cutoff, Spectrum Medical Industries, Los Angeles, CA). The dialyzed medium was lyophilized and reconstituted in 2 ml of deionized water. Two ml of ^{125}I-h_rIGF-I (1.0×10^6 cpm/ml; see chapter 2 for a description of iodination procedure) was added to the concentrated medium and incubated for 20 h at 4°C. One ml of this mixture was removed and added to one ml 0.94 M sucrose dissolved in column buffer (50 mM Tris-HCl, 0.2 M NaCl, 0.01% thimersol, pH 7.4). The sample was chromatographed on Sephacryl S-200 equilibrated in column buffer as described in chapter 2. Another aliquot (1 ml) of the labeled medium was treated with glycylglycine-HCl (pH 2.0, 170 ul). The acidified medium was incubated for 48 h at room temperature and chromatographed on Sephacryl S-200 as described above for the untreated sample.

Statistical Procedures. Data were analyzed by standard analysis of variance techniques as described by Steel and Torrie (99) using a computer program for unbalanced treatment replications (MACE Inc., 1984). When significant main effect and/or interaction were noted, means were separated using Student's t-test criterion.

RESULTS

pGH was elevated 4-5 fold in the sera of neonatal pigs treated with pGH compared to control pigs (table 1). No differences were found in serum pGH levels between the three treatment groups comparing controls and treated animals. Despite elevated levels of pGH in sera, treated pigs did not exhibit increased rates of growth or greater body weights than their age-matched control littermates (table 2). As expected, animals in group I had lower final body weights than group II and III animals because the former were 1 wk younger. In addition, IGF-I levels in sera from piglets treated with pGH were not different from control levels in any of the treatment groups (table 3).

The viability of hepatocytes, as measured by trypan blue exclusion, isolated from control and pGH treated neonatal pigs was similar (79 ±5 overall mean ±SD; N=62). Initial results indicated that primary monolayer cultures of hepatocytes that were maintained in serum-free medium secreted low levels of IR-IGF-I during the 24 hour period. In some samples the quantity approached the detection limits of the assay (0.1 ng/ml). Hepatocytes isolated from piglets obtained from treatment group III were used for preliminary assessment of the effects of pGH on IR-IGF-I synthesis. Since prior treatment of piglets with pGH did not affect the basal level of IGF-I synthesis by isolated hepatocytes (table 4), data from hepatocytes prepared from control and treated pigs were combined to examine the

effect of pGH on the synthesis of IR-IGF-I *in vitro*. IR-IGF-I synthesis was not affected by pGH at concentrations of 50-500 ng/ml in the culture medium (table 5). Although these results were obtained from a limited number of animals, they indicate that IR-IGF-I synthesis in neonatal hepatocytes is not regulated by GH. Together with the lack of effect of pGH *in vivo*, these results suggest that growth in the neonatal pig occurrs independently of GH.

The possibility that isolated neonatal hepatocytes were secreting carrier proteins was also investigated. In a preliminary experiment, serum-free medium conditioned by hepatocytes was concentrated and incubated with ^{125}I-labeled h_rIGF-I. When this sample was fractionated on Sephacryl S-200 equilibrated in neutral buffer, approximately 65% of the recovered radioactivity eluted in a high molecular weight region (K_d=0.2-0.4, figure 1). The remainder of the radioactivity eluted at a position similar to free ^{125}I-h_rIGF-I (K_d=0.5-0.7). When an identical sample was treated with glycylglycine-HCl for 48 h and chromatographed under the same conditions, almost all of the radioactivity eluted as free hormone (figure 2). These results indicate that hepatocytes synthesize material capable of binding IGF-I and that this complex is acid-labile. In addition, the molecular weight of this material was similar to the binding species present in neonatal sera (see chapter 2).

TABLE 1. EFFECT OF pGH TREATMENT ON SERUM pGH LEVELS OF NEONATAL PIGS.

Treatment[1] group	Serum pGH	
	Control	pGH-Treated
	ng/ml	
I	4.1 ±0.7[a]	18.2 ±2.0[b]
II	4.2 ±0.8[a]	18.5 ±0.8[b]
III	4.6 ±0.6[a]	22.6 ±2.3[b]

[a,b]Values are given as mean ±SEM Values with different superscripts are different, $p<0.001$.

[1]Daily pGH injections (50 $ug \cdot kg^{-1} \cdot BWt^{-1}$) were initiated when piglets were either 3-7 days of age (groups I and III) or 10-14 days of age (group II) and continued for either one week (groups I and II) or two weeks (group III). In each group, there were 9 control and 9 pGH-treated piglets.

TABLE 2. EFFECT OF pGH TREATMENT ON FINAL BODY WEIGHT AND AVERAGE DAILY GAIN OF NEONATAL PIGS.

Treatment[1] group	Final Body Weight		Average Daily Gain	
	Control	pGH Treated	Control	pGH Treated
	kg		g/day	
I	4.0 ±0.2[a]	4.0 ±0.2[a]	232 ±13[c]	239 ±17[c]
II	5.6 ±0.3[b]	5.2 ±0.3[b]	208 ±22[c]	216 ±18[c]
III	5.3 ±0.3[b]	5.0 ±0.3[b]	206 ±19[c]	197 ±16[c]

[a,b,c]Values are given as mean ±SEM Values for body weight and average daily gain with different superscripts are different, $p<0.01$.

[1]Treatment groups are as described in table 1 except that there were 14 control and treated animals for groups I and II, and 13 control and treated animals for group III.

TABLE 3. EFFECT OF pGH TREATMENT OF NEONATAL PIGS ON SERUM IGF-I LEVELS

Treatment[1] group	Serum IGF-I	
	Control	pGH Treated
	ng/ml	
I	128 ±12[a]	140 ±18[a]
II	115 ±20[a]	119 ±22[a]
III	96 ±15[a]	139 ±17[a]

[1]Treatment groups are as described in table 1.

[a]Values are given as mean ±SEM There were no significant effects of pGH treatment or group, $p>0.10$.

TABLE 4. EFFECT OF IN VIVO TREATMENT WITH pGH ON IN VITRO SYNTHESIS OF IR-IGF-I BY NEONATAL SWINE HEPATOCYTES[1].

Treatment	IR-IGF-I
	ng/mg protein/24 h
Control	0.5 ±0.2[a]
pGH Treated	0.6 ±0.2[a]

[1]Hepatocytes were isolated from control and pGH treated neonatal swine (group III) as described in "MATERIALS AND METHODS". Serum-free medium conditioned between 48 and 72 h in culture was collected and analyzed for IGF-I as described in "MATERIALS AND METHODS".

[a]Values represent mean ±SEM of 3 animals per treatment. Conditioned medium from duplicate culture dishes was analyzed for each animal.

TABLE 5. EFFECT OF IN VITRO TREATMENT OF NEONATAL SWINE HEPATOCYTES WITH pGH ON IR-IGF-I SYNTHESIS[1].

Dose of pGH added	IR-IGF-I
ng/ml	ng/mg protein/24 h
0	0.6 ±0.1[a]
50	0.6 ±0.2[a]
250	0.7 ±0.1[a]
500	0.7 ±0.2[a]

[1]Hepatocytes were isolated from neonatal piglets (group III) as described in "MATERIALS AND METHODS". Data from control and pGH treated animals were combined. pGH was added to serum-free medium to hepatocytes at 48 h in culture. After 24 h, conditioned medium was collected and analyzed for IGF-I content as described in "MATERIALS AND METHODS".

[a]Values are given as mean ±SEM of 6 animals. Conditioned medium from duplicate culture dishes was analyzed for each animal. There were no differences between media from cultures exposed to different levels of pGH, $p>0.10$.

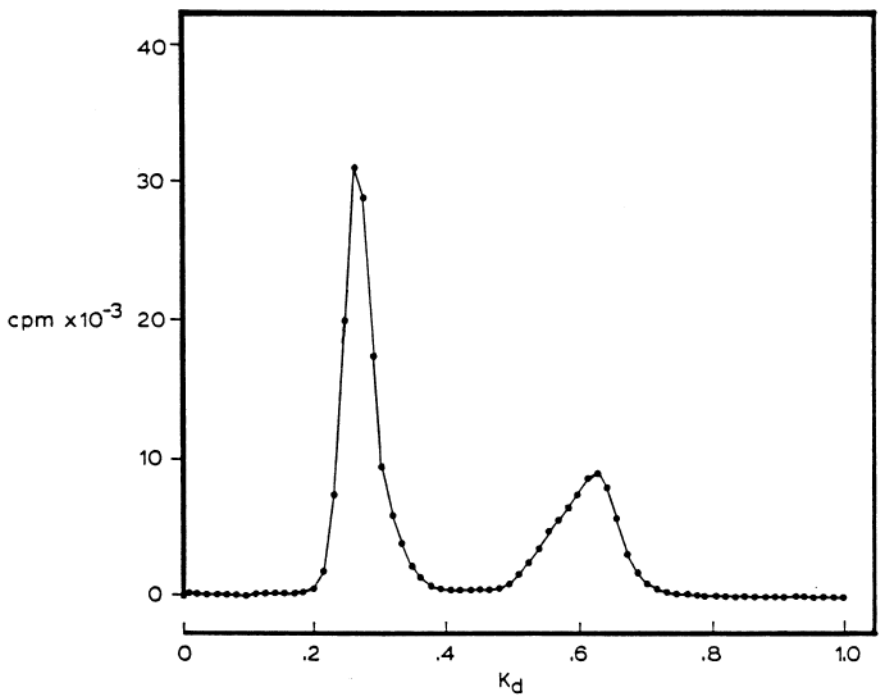

Figure 1. Elution profile of conditioned medium fractionated on Sephacryl S-200 after labeling with ^{125}I-h_rIGF-I. Conditioned medium was dialyzed, concentrated, and labeled with ^{125}I-h_rIGF-I as described in "MATERIALS AND METHODS". The sample was chromatograghed on Sephacryl S-200 that had been equilibrated in 50 mM Tris-HCl buffer, pH 7.4 as described in chapter two.

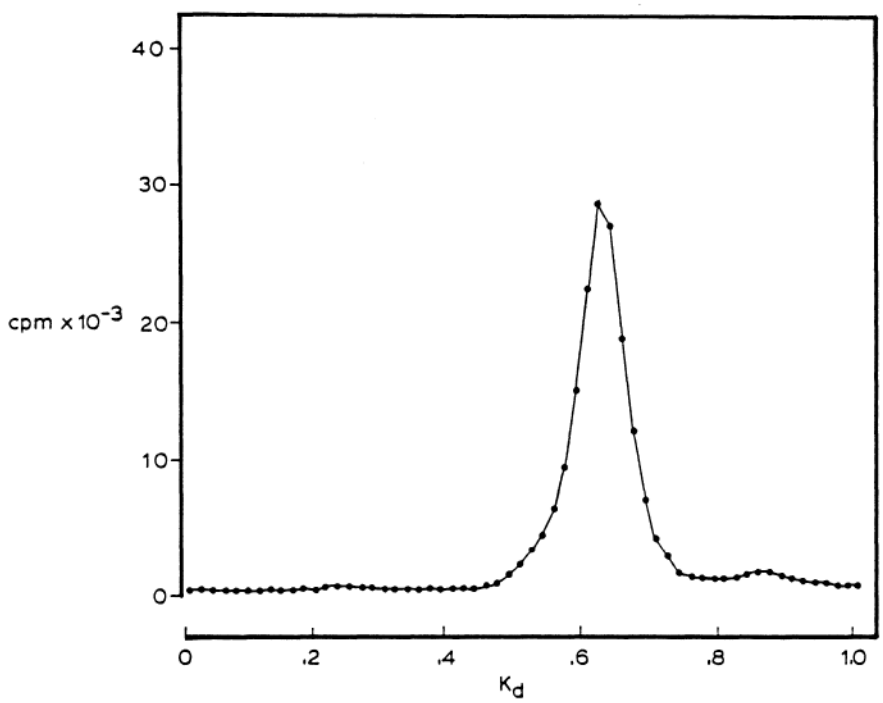

Figure 2. Effect of glycylglycine-HCl treatment on the elution profile of labeled culture medium fractionated on Sepacryl S-200. Conditioned medium that had been labeled with ^{125}I-h$_r$IGF-I was treated with 0.2 M glycylglycine-HCl (final pH 3.6) for 48 h and then fractionated on Sephacryl S-200 as described in figure 1.

DISCUSSION

The effects of chronic pGH administration to rapidly growing pigs between 30 and 90 kg BWt have been previously investigated (1-3,89). Doses of pGH between 20 and 70 ug·kg $BWt^{-1}\cdot day^{-1}$, administered daily for 35 days, significantly improved growth rate, feed efficiency, and carcass composition (3). The effect of pGH treatment of neonatal swine on growth has not been previously investigated. The experimental design used in this study necessitated consideration of several unique characteristics of the newborn pig. Newborn piglets have a limited capacity for gluconeogenesis due to incomplete development of this metabolic pathway (100). Because of this, the hepatic glycogen stores present at birth are rapidly depleted during early postpartum period (100). By 5 d of age this pathway is fully developed in neonatal pig liver (101). Furthermore, there is evidence that the newborn pig has a limited capacity for energy production due to a deficiency of hepatic mitochondrion (102). Because of these immature metabolic capacities in the newborn, pGH treatment was not initiated until 3-7 d postpartum. In addition, there is evidence that hepatic GH receptors are low in the fetal and neonatal lamb and do not increase to adult levels until after 6 d of age (21). Therefore, a second group of piglets was treated with pGH beginning at 10 d of age to offset possible influences of age on GH receptors in liver.

Despite elevations of pGH in sera from neonates treated with pGH, growth rate was not affected in these animals. The levels of pGH attained in neonatal sera were comparable with those obtained by other investigators (1). Chung and coworkers (1) found that treatment with pGH did not affect growth rate until after 21 days. Since the longest period of treatment in this study was 14 days, it is possible that treatment for longer periods might affect growth rate. However, the finding that IGF-I levels in sera from the neonatal pigs were unchanged argues against this possibility. Recently, Sillence et al. (103) found that sera levels of IGF-I began to rise approximately 4 h after treatment of weaned pigs (58 kg) with a single injection of GH (100 or 1000 ug/kg bwt) and remained elevated for 36 h. If neonatal pigs responded in a manner similar to weaned pigs, one or two weeks of treatment with pGH should have been sufficient to elicit an IGF-I response. Two explanations of these data are possible. Either growth in neonatal swine is not regulated by GH or growth is GH-dependent but, endogenous levels of GH are sufficient for neonatal pigs to grow at a maximal rate. Although *in vivo* data from this study do not distinguish between these two possibilities, several findings indicate that growth of neonatal swine is probably GH-independent.

Fetal growth proceeds normally in the absence of the pituitary in man (17), monkey (18) and rabbit (19), suggesting that growth *in*

utero occurs independently of GH. Furthermore, Walker and coworkers (20) found that the onset of GH-dependent growth was gradual in HYPOX neonatal rats between 10 and 28 d of age. The finding that GH binding to liver microsomes prepared from sheep (21) and rats (22) increases from birth is consistent with a gradual dependence of growth on GH. Correlated with a low binding of GH to liver microsomes at birth is the finding that IGF-I levels are low in sera from neonates of several species. In one study, IGF-I levels in sera from fetal rats were 70% of adult values and then decreased between birth and 18 d of age to 15% of adult values, and finally, increased to about 57% of adult levels by 25 d (41). In contrast, Sara et al. (90) found that somatomedin A (identical to IGF-I; 104) levels in fetal sera were only 5% of adult values and increased to approximately 60% of adult levels by 25 d. Daughaday et al. (41) attributed this difference to interference of carrier proteins in the RIA employed by Sara et al. (90). In mice, the levels of IGF-I in sera remained low throughout the fetal and neonatal period, and then started to increase at 4 wk of age (48).

In the present study, the average IGF-I concentration of all neonatal sera examined (10-21 d of age, N=54) was 123 ±7 (mean ±SEM). This value is about 40% less than the adult value measured in pooled sera prepared from 60 kg pigs. This result suggests that IGF-I levels in swine sera also increase with age after birth. In contrast to IGF-I, IGF-II levels in sera prepared from fetal rats

were markedly elevated compared to maternal sera and then gradually decreased to adult levels by 25 d of age (42). Together, these results suggest that in swine there is a shift from GH-independent growth *in utero* (possibly mediated by IGF-II) to GH-dependent growth post-weaning (mediated by IGF-I).

Ultrafiltration was shown to be a rapid and effective procedure for separation of IGF-I from its carrier proteins (chapter 2). The importance of removing the carrier proteins before RIA of IGF-I can be appreciated when it is realized that carrier proteins are also regulated developmentally (91,92). White et al. (92) have shown that in rats during fetal and early neonatal life the 30-45 kDa GH-independent IGF-I carrier protein was predominant in sera. The 150 kDa GH-dependent IGF-I carrier protein complex became the major IGF binding species after growth became GH-dependent (92). In addition, Draznin et al. (91) have used a competitive binding assay for carrier proteins to show that the binding capacity of neonatal rat sera for IGFs increased 6-fold between 1 and 41 d of age. Thus, Daughaday et al. (41) suggested that the rise in serum IGF-I measured by Sara et al. (90) in the neonatal rat between 1 and 50 d of age actually reflected a rise in carrier protein levels.

A preliminary series of experiments were performed to examine the synthesis of IR-IGF-I by primary cultures of hepatocytes prepared from neonatal swine treated *in vivo* with pGH. The results indicated that *in vivo* treatment of piglets with pGH had no effect on the

synthesis of IR-IGF-I by isolated hepatocytes. Furthermore, the direct addition of pGH to hepatocytes in vitro also had no effect on synthesis of IR-IGF-I. These results are consistent with the lack of effect of pGH treatment in vivo on growth rate. Although there are no other reports on examination of IGF-I synthesis in neonatal hepatocytes, some results from other species may be useful in explaining these findings. As metioned above, the finding that somatogenic (GH) binding sites in sheep and rats are low at birth and gradually increase with age implies that liver responsiveness to GH is developmentally regulated (21,22). It is possible that the liver from 10-21 d old piglets did not respond to pGH treatment because of a deficiency in GH receptors. In contrast to the pig, GH binding to liver of sheep (21) and rats (22) increased to adult levels at 6 and 8 d, respectively, after birth.

Richman and coworkers (69) have observed that fetal rat hepatocytes in primary culture produce primarily an IGF-II-like peptide. These investigators employed the antibody utilized in this study to measure IR-IGF-I synthesis in fetal hepatocytes. The levels they detected were approximately 500-fold lower than the somatomedin activity they measured using a competitive protein binding assay that measures both IGF-I- and IGF-II-like peptides. They concluded that the IR-IGF-I they measured was really IGF-II that cross-reacted with the IGF-I antisera. In addition, the synthesis of IGF-II was minimally affected by the addition of rat GH to the cultures. In

contrast, hepatocytes isolated from adult rats primarily produce IGF-I in culture (68,105,106). Furthermore, adult rat hepatocytes responded to bovine GH with a 38% increase in the rate of IGF-I synthesis (107). Therefore, one can surmise from these data that neonatal porcine hepatocytes may not have developed the ability to respond to GH in vitro. The finding that the levels of IR-IGF-I produced by porcine hepatocytes are approximately 55-fold less than the levels reported for adult rat hepatocytes (107) supports this hypothesis. The possibility that neonatal porcine hepatocytes are producing IGF-II requires further studies using assays specific for this peptide.

The synthesis of carrier protein(s) by the liver has been well documented (63,64,67-69,105-107). Although the question of whether the liver secretes more than one carrier protein in vivo has not been resolved, Scott et al. (68) have found that hepatocytes isolated from adult rats secrete a binding species which contained a single class of binding sites and had an indicated molecular weight of 50 kDa. However, definitive proof that this binding protein is a single molecular species is lacking. In addition, other cell types including fibroblasts (95) and fetal myoblasts (94) produce carrier proteins for IGFs. In fact, there have been no reports of a cell type that has synthesized IGF without the simultaneous production of a carrier protein (13). Consequently, the possibility that neonatal hepatocytes were producing a carrier protein was investigated.

Medium conditioned by neonatal porcine hepatocytes contained a species that bound labeled h_rIGF-I and had an apparent molecular weight of 35-45 kDa. The labeled hormone was completely displaced from these binding sites by exposure to glycylglycine-HCl at pH 3.6 before fractionation by gel filtration chromatography. Whether this binding is specific for IGF-I requires further investigation.

The level of carrier protein(s) synthesis can be estimated from th binding of labeled h_rIGF-I if several assumptions are made. The specific activity of the labeled h_rIGF-I was 200 cpm/pg and was decreased to 91 cpm/pg by endogenous IGF-I. The value was derived from a measured concentration of 0.65 ng IGF-I/mg protein in the medium (table 5) or 6.05 ng IGF-I in total sample to which labeled peptide was added. If the amount of radioactivity that eluted as a complex is summed (2.26×10^5 cpm) and divided by the specific activity and the molecular weight of IGF-I (7.85 kDa), then approximately 314 femtomoles (fm) of IGF-I were present in this peak. Without knowing the stoichiometry between the binding species and IGF-I, this equates to about 34 fm IGF-I binding sites/mg protein/24 h. Since the rate of synthesis of IR-IGF-I was about 83 fm/mg protein/24 h (table 5) and assuming that 1 mole of IGF-I binds to 1 mole of carrier protein(s), the level of carrier protein(s) synthesis was similar to the rate of IR-IGF-I synthesis. These data provide preliminary evidence that carrier protein(s) and IGF-I synthesis were coordinately regulated in primary monolayer cultures

of neonatal hepatocytes.

In summary, treatment of neonatal pigs with pGH did not affect growth rate. IGF-I levels in neonatal swine sera have been measured for the first time using an antisera specific for human IGF-I and a method which quantitatively separates IGF-I from carrier proteins, viz., ultrafiltration. IGF-I levels in neonatal swine sera were 40% lower than that in pooled sera from adult swine. In addition, pGH treatment of neonatal pigs had no effect on circulating levels of IGF-I. Preliminary investigations with neonatal swine hepatocytes in culture revealed that IR-IGF-I synthesis was not dependent on pGH. Finally, neonatal hepatocytes synthesize a species capable of binding labeled h_rIGF-I that resembles an IGF carrier protein. The level of IGF carrier protein(s) synthesis was of the same order of magnitude as IR-IGF-I synthesis. Together these results indicate that growth, or more specifically, IGF-I production is not inducible by pGH in the neonatal pig.

SUMMARY AND CONCLUSIONS

An antisera specific for human IGF-I was used to measure IGF-I in swine. Since swine carrier proteins were shown to interfere in the RIA following acidic treatment of samples, a method based on ultrafiltration was used to quantitatively separate carrier proteins from IGF-I. Ultrafiltration had several advantages over previous separation methods, viz., gel filtration chromatography and HPLC. The most important benefits were a capacity to process large numbers of samples (48 per day) and ease of operation. Quantitative spearation was evidenced by a lack of IGF-I binding material in the ultrafiltrate. In addition, the recovery of added h_rIGF-I from swine serum was linear and averaged 72%. Furthermore, swine chronically treated with pGH had 2.6-fold higher sera levels of IGF-I than control samples. In contrast, serum from a HYPOX animal was only 4% that of normal animals.

This procedure was then applied to the study of the relationship between GH and IGF-I in the neonatal pig. Neonatal pigs were treated with pGH for 1 or 2 wk beginning at 3 to 7 d of age or for 1 wk beginning at 10 to 14 d of age. Despite 4 to 5-fold elevations in serum GH of treated piglets, growth and serum IGF-I levels were not affected. Preliminary results from primary monolayer cultures of neonatal swine hepatocytes maintained in serum-free medium indicated that IR-IGF-I synthesis was not regulated by GH. In addition,

isolated hepatocytes synthesized a specie(s) capable of binding IGF-I that had a molecular weight similar to a carrier protein noted in swine sera. The amount of this binding specie(s) secreted by isolated hepatocytes was similar to the amount of IR-IGF-I secreted by these cells suggesting that IGF-I and carrier protein(s) may be coordinately regulated in neonatal hepatocytes.

These results suggest that either growth in neonatal swine is not regulated by GH or growth is GH-dependent but endogenous levels of GH are sufficient for piglets to grow at a maximal rate. Since growth at the molecular level is poorly understood, it is not unlikely that growth factors in addition to GH and IGF-I may be important in regulating growth in the neonatal pig.

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