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### Research Article

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With this assay it is possible to detect somatomedin-C in normal subjects using as little as 0.25  $\mu\text{l}$  of unextracted serum. Serum somatomedin-C concentrations in normal subjects were lowest in cord blood and rose rapidly during the first 4 yr of life to near adult levels. In 23 [...]

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# Estimation of Somatomedin-C Levels in Normals and Patients with Pituitary Disease by Radioimmunoassay

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**ABSTRACT** The development of a radioimmunoassay for somatomedin-C has for the first time made it possible to discriminate between serum concentrations of a single peptide or closely related group of peptides and the net somatomedin activity measured by less specific bioassay and radioreceptor techniques. Antibodies to human somatomedin-C were raised in rabbits using a somatomedin-C ovalbumin complex as the antigen. A variety of peptide hormones at concentrations up to 1  $\mu$ M are not recognized by the antibody. Insulin at concentrations  $>0.1 \mu$ M cross reacts in a non-parallel fashion; purified somatomedin-A is only 3% as active as somatomedin-C; and radiolabeled cloned rat liver multiplication stimulating activity does not bind to the antibody. Immunoreactive somatomedin-C can also be quantitated in the sera of a variety of subhuman species.

Unusual assay kinetics, which are manifest when reactants are incubated under classic "equilibrium" assay conditions, appear to result from the failure of  $^{125}$ I-somatomedin-C to readily equilibrate with the somatomedin-C serum binding protein complex. It is, therefore, necessary to use nonequilibrium assay conditions to quantitate somatomedin-C in serum.

With this assay it is possible to detect somatomedin-C in normal subjects using as little as 0.25  $\mu$ l of unextracted serum. Serum somatomedin-C concentrations in normal subjects were lowest in cord blood and rose rapidly during the first 4 yr of life to near adult levels. In 23 normal adult volunteers, the mean serum

somatomedin-C concentration was  $1.50 \pm 0.10$  U/ml (SEM) when compared to a pooled adult serum standard. 19 children with hypopituitary dwarfism had concentrations below 0.20 U/ml. 17 of these were below 0.1 U/ml, the lower limit of sensitivity of the assay. The mean concentration in 14 adults with active acromegaly was  $6.28 \pm 0.37$  U/ml (SEM), five times greater than the normal volunteers. Significant increases in serum somatomedin-C concentrations were observed in 8 of 10 hypopituitary children within 72 h after the parenteral administration of human growth hormone. Three patients with Cushing's disease had elevated serum somatomedin-C concentrations ( $2.61 \pm 0.14$  U/ml [SEM]). Three patients with hyperprolactinemia had normal concentrations ( $1.74 \pm 0.11$  U/ml [SEM]).

The important new discovery brought to light by quantitation of immunoassayable somatomedin in patient sera is that all previously used assays detect, in addition to somatomedin-C, serum substances that are not under as stringent growth hormone control.

## INTRODUCTION

Many of the physiological effects of growth hormone are now believed to be mediated through the somatomedins. These substances are characterized by three cardinal properties: their concentrations in serum are regulated by growth hormone, they stimulate sulfate incorporation into cartilage, and they possess intrinsic insulinlike activity (1). Four substances fulfilling these criteria have been described: nonsuppressible insulinlike activity, multiplication stimulating activity, somatomedin-A, and somatomedin-C.<sup>1</sup> The identity or

<sup>1</sup> Somatomedin-B, although under growth hormone control, is no longer considered a somatomedin because it does not stimulate sulfate uptake into cartilage or have insulinlike activity.

A preliminary report of this work was presented at the 5th International Congress of Endocrinology, Hamburg, Federal Republic of Germany, 18-24 July 1976.

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lack of identity among these substances remains to be established.

Originally, these peptides could be quantitated by bioassays based on sulfate or thymidine uptake by cartilage (2, 3, 3a), by insulinlike activity in nonskeletal tissues (4), or by growth promoting activity in cultured cells (5). More recently the availability of small amounts of pure hormone suitable for iodination has led to the development of competitive membrane binding assays for these substances (6, 7, 7a). Inasmuch as the somatomedins have qualitatively similar biologic actions, it is not surprising that each cross reacts with the others in their respective competitive membrane binding assays (8, 9). More specific assays are, therefore, required to determine the physiological importance of the individual somatomedins and to study their roles in disease states.

This report describes the development of a highly sensitive and specific radioimmunoassay for somatomedin-C. Results obtained with this more specific technique suggest that somatomedin-C itself is under stricter growth hormone control than are some of the other substances contributing to the net somatomedin activity measured by biological or cell membrane receptor assays. Initial difficulties encountered in measuring this hormone in unextracted serum were attributed to the presence of a serum binding protein for somatomedin-C. The successful resolution of this difficulty was based on kinetic considerations which may have generalized relevance to other radioligand assays carried out in the presence of binding proteins.

## METHODS

**Materials.** Porcine insulin (615-D63-10) and glucagon (258-D30-1384) were gifts from Eli Lilly and Company (Indianapolis, Ind.). Nerve growth factor, epidermal growth factor, and pituitary fibroblast growth factor were gifts of Doctors Ralph Bradshaw (Washington University School of Medicine, St. Louis, Mo.), Stanley Cohen (Vanderbilt University School of Medicine, Nashville Tenn.), and Denis Gospodarowicz (Salk Institute, La Jolla, Calif.), respectively. Rat somatomedin was kindly supplied by Dr. William Daughaday (Washington University School of Medicine, St. Louis, Mo.). Purified somatomedin-A (SPE 152-1) was a gift of Dr. Linda Fryklund, AB Kabi, (Stockholm, Sweden). <sup>125</sup>I multiplication stimulating activity obtained from cloned rat liver cells was provided by Dr. S. Peter Nissley (National Cancer Institute, Bethesda, Md.). Human urinary erythropoietin (H4H-35) was supplied by the National Lung Institute. Ovine prolactin (P-S-9), ovine follitropin (S-10), ovine lutropin (S-18), human somatotropin (HS 1395), and bovine thyrotropin (B-6) were provided by the Hormone Distribution Officer of the National Institute of Arthritis and Metabolic Diseases and the National Pituitary Agency (University of Maryland). ACTH 1-24 (Cortrosyn, lot 274731B) was obtained from Organon Inc. (West Orange, N. J.). Lysine vasopressin (grade IV, 65 C-0156) and ovalbumin (A5503, lot 33C-8345) were purchased from the Sigma Chemical Co. (St. Louis, Mo.). Gluteraldehyde, 50% in water, was purchased from Eastman

Organic Chemicals, Div. of Eastman Kodak Co. (Rochester, N. Y.).

**Purification of somatomedin-C and preparation of the somatomedin-C ovalbumin complex.** Cohn fraction IV prepared from fresh frozen human plasma was extracted with phosphoric acid and then concentrated by cation exchange chromatography on SP Sephadex in ammonium formate buffer. Active fractions were further concentrated in an Amicon ultrafiltration cell (Amicon Corp., Scientific Sys. Div., Lexington, Mass.) and then chromatographed on Sephadex G-50 in formic acid.<sup>2</sup> The active fractions from the Sephadex G-50 column were further purified by sequential isoelectric focusing, first over a broad range (pH 3.5–pH 10) and then over a narrow range (pH 8.0–pH 10.0). The somatomedin used to prepare the immunogen contained material which isofocused above pH 8 and was free of immunoreactive insulin. This preparation was judged to be 14% pure.

The antigen used for immunization was prepared by conjugating somatomedin-C to ovalbumin using gluteraldehyde as the coupling agent (10). 1 mg of ovalbumin and 2.9 mg of the partially purified preparation of somatomedin (containing 420 μg of somatomedin) were dissolved in 0.70 ml of 0.10 M phosphate buffer, pH 7.41. To this solution, 0.30 ml of 0.13 M gluteraldehyde in phosphate buffer was added dropwise with stirring at 23°C. During the addition of gluteraldehyde, the solution turned pale yellow. The reaction was allowed to proceed at room temperature for 14 h. The reaction mixture was then dialyzed extensively against 0.15 M sodium chloride.

**Production of antiserum.** Four mature New Zealand white rabbits weighing approximately 4 kg each were immunized by the multiple site immunization procedure described by Vaitukaitis et al. (11) except that *Bordetella pertussis* vaccine was omitted. In the primary injection, each animal received 625 μg of the somatomedin-C ovalbumin complex, corresponding to 70 μg of somatomedin-C. 6 wk later, each rabbit with detectable somatomedin antibodies was rechallenged with 100 μg of the somatomedin-C ovalbumin complex (11 μg somatomedin-C) emulsified in incomplete Freund's adjuvant and given subcutaneously at four sites. The rabbits were bled from the central ear artery just before the primary immunization and approximately biweekly thereafter.

**Somatomedin standard.** The somatomedin reference standard (MUC) is a human serum pool obtained from three healthy adult volunteers and arbitrarily defined as containing 1 U of somatomedin activity/ml. This standard has been used in our laboratory for the last 2 yr as a reference preparation for the somatomedin competitive membrane receptor assay (6).

**Conditions used for radioimmunoassay.** The preparation of somatomedin-C used for iodination was isolated in our laboratory by procedures described previously (12). It contains approximately 5,800 U/mg and <1 part in 10,000 of immunoreactive insulin. This preparation was approximately 90% pure as judged by sequential Edman degradation. This material was iodinated by the fractional chloramine-T method and the iodinated hormone was purified on G-50 Sephadex (8). Before use, free iodine was removed by adsorption with an ion exchange resin (Bio-Rad AG1-X8; BioRad Laboratories, Richmond, Calif.). Assays were carried out at 4°C in 0.03 M phosphate buffer, pH 7.4 containing

<sup>2</sup> These steps were performed at Armour Laboratories, Kankakee, Ill., under the direction of Dr. R. Schlueter. Details of this procedure will be reported subsequently (Van Wyk, J. J., C. J. Decedue, and R. J. Schlueter, to be published).

0.25% bovine serum albumin and 0.02% sodium azide. All reagents were added with a Micromedic automatic pipette (Micromedic Systems, Inc., Horsham, Pa.). Samples were assayed in duplicate at two or more concentrations. Final volume of the reaction mixture was 0.50 ml. Bound and free hormone were separated by double antibody precipitation with sheep anti-rabbit globulin.

Two methods of assay, which differed in the order of addition of reagents, were employed. In the first, graded amounts of unlabeled somatomedin or unknowns were mixed with  $^{125}\text{I}$ -somatomedin-C (approximately 0.75 fmol; 15,000 cpm), and the reaction was initiated by the addition of antiserum. The sample was incubated for 88 h at 4°C after which the reaction was terminated by the addition of a second antibody. This order of reagent addition is the standard or "equilibrium" assay procedure usually employed in radioimmunoassay systems.

In the second method of assay, the standard or unknown was added to the assay tube in a volume of 200  $\mu\text{l}$ . This was followed by the addition of 150  $\mu\text{l}$  of an appropriate dilution of somatomedin antiserum. After incubation at 4°C for 72 h,  $^{125}\text{I}$ -somatomedin-C was added in 150  $\mu\text{l}$  of buffer. After an additional 16 h of incubation, the reaction was terminated by the addition of the second antibody. This order of reagent addition, which has been termed the "non-equilibrium" assay procedure, has been used as a means of increasing sensitivity in competitive protein binding assays (13). The 72-h preincubation time was adopted after studies showed that this interval was necessary for equilibrium to occur.

*Competitive membrane binding assay for somatomedin.* The competitive membrane binding assays for somatomedin were performed with a crude placental cell membrane preparation as previously described (6, 8).

*Gel filtration of serum at neutral pH.* 40 ml of normal human serum were incubated with  $^{125}\text{I}$ -somatomedin-C (300,000 cpm; approximately 15 fmol) for 6 h at 5°C. The mixture was then chromatographed on a 5  $\times$  95-cm column of Sephadex G-200 equilibrated in 0.05 M phosphate buffer, pH 7.6. The column was developed in an ascending fashion at 4°C at 25 ml/h. After removal of aliquots for determination of radioactivity, fractions corresponding to approximately 0.05  $K_{av}$  intervals were combined and assayed.

*Gel filtration of serum and of partially purified somatomedin in 1 M acetic acid.* Separation of somatomedin-C from its binding protein was accomplished by gel filtration of 0.5 ml of serum on a 1.2  $\times$  47.5-cm column of Sephadex G-50 (medium) equilibrated in 1 M acetic acid, pH 2.48 containing 0.02% sodium azide. The column was eluted at 5°C by descending chromatography at 1 ml/min. The void and inclusion volumes were determined with rabbit IgG and sodium chloride, respectively. Fractions corresponding to the desired  $K_{av}$  intervals were pooled, lyophilized to dryness, and resuspended in appropriate volumes of assay buffer.

*Patient selection and study methods.* Although no attempt was made to standardize the conditions or time of blood specimen collection, most samples were obtained between 10:00 and 16:00 h. The group of normal children ranged in age from 10 mo to 16 yr. These children were within 2 SD of the mean height for age and were free of clinical evidence of serious illness. Cord sera were obtained from 10 healthy, normal-sized term infants. The group of normal adult subjects, composed of 23 healthy volunteers (13 male, 10 female) ranged in age from 19 to 55 yr. None was receiving medication at the time of sampling. The 14 patients with active acromegaly had elevated basal serum concentrations of immunoreactive growth hormone which did not suppress

below 6 ng/ml after the oral administration of a 100 g glucose load. Some of these patients had been treated previously but showed evidence of persistence of their growth hormone excess. The 19 hypopituitary patients, ranging in age from 5 to 24 yr, have been described in detail elsewhere (14). Included in this group are patients with isolated growth hormone deficiency, idiopathic panhypopituitarism, and hypopituitarism secondary to central nervous system tumors.

Two of the three patients with Cushing's disease due to pituitary ACTH excess have been reported previously (15). The three patients with hyperprolactinemia all had elevated immunoassayable serum prolactin levels (>100 ng/ml).<sup>3</sup> Two of these patients had surgically proved pituitary adenomas, whereas the third had no radiologic evidence of a pituitary tumor. The growth hormone response to insulin was normal in the two patients in whom adequate hypoglycemia was obtained.

Studies on the induction of somatomedin by the administration of human growth hormone in previously untreated hypopituitary patients were performed as previously described (14). Blood samples were obtained on the 10 study patients on 2 successive days before the administration of human growth hormone. Human growth hormone, 1 U, was administered intravenously at time 0. This was followed by 1 U intramuscularly at 6, 24, 48, and 72 h. Blood samples were obtained for somatomedin concentration at 0, 0.5, 1, 3, 6, 12, 24, 48, and 72 h.<sup>4</sup>

## RESULTS

*Characteristics of the antiserum to somatomedin.* Antibodies to somatomedin were detectable in the serum of three of the four immunized rabbits 28 days after the primary injection of antigen. After challenge with the somatomedin-C ovalbumin complex, each of these animals had a significant rise in antibody titer. On the 48th day after the primary immunization, an antiserum was obtained which bound 70% of the  $^{125}\text{I}$  somatomedin-C at a 1:10,000 dilution. This antiserum (2574) has a  $K_d$  of  $4.6 \times 10^{10}$  liters/mol, and each milliliter is capable of binding 0.5 nmol of somatomedin (16). Although the three responding rabbits had prolonged anamnestic responses and the antisera obtained from later bleedings had higher titers, the studies reported here were performed with antiserum 2574 at a final dilution of 1:10,000.

*Characteristics of the radioimmunoassay under equilibrium and nonequilibrium conditions.* With the equilibrium assay procedure, the competitive dose-response curves for the serum standard were not parallel to those of the partially purified hormone (Fig. 1). Particularly striking was the finding that at high serum concentrations the competitive dose-

<sup>3</sup> We thank Dr. Jerome Feldman of the Department of Medicine, Duke University, for providing us with many of the above samples.

<sup>4</sup> Human growth hormone was provided by the National Institute of Arthritis, Metabolism, and Digestive Diseases and the National Pituitary Agency of the University of Maryland; and by AB Kabi, Stockholm, Sweden.

response curve plateaued at a  $B/B_0$  value well above that observed for partially purified somatomedin. This behavior, however, was not observed when the somatomedin activity was extracted from serum by gel filtration in 1 M acetic acid before assay.

By using the nonequilibrium assay method, the competitive dose-response curve for unextracted serum samples and for partially purified somatomedin were superimposable (Fig. 2). Moreover, the nonequilibrium assay procedure abolished the plateau effect observed at high concentrations of serum. A possible explanation for the differences observed with the two assay methods will be discussed. Once these differences were known all subsequent assays were performed by the nonequilibrium technique.

With the nonequilibrium method, as few as  $5 \times 10^{-4}$  U/ml of reaction mixture can be detected and 50% displacement is observed with  $1.8 \times 10^{-3}$  U of somatomedin/ml. Intra-assay variability, expressed as the coefficient of variation, is 5% for sera from normal subjects, 10% for acromegalic samples, and as high as 20% for hypopituitary samples. Interassay variability, determined by assaying the same samples in 10 consecutive assays and expressed as the coefficient of variation, was approximately 10% for normal and acromegalic samples, and 25% for samples from hypopituitary patients.

**Hormonal specificity.** Detailed assessment of the effect of incremental concentrations of a variety of hormones indicated that the somatomedin-C antiserum is highly specific. No inhibition of  $^{125}\text{I}$ -somatomedin-C binding was observed with a variety of growth factors, pituitary hormones, or ovalbumin (Fig. 3). Bovine

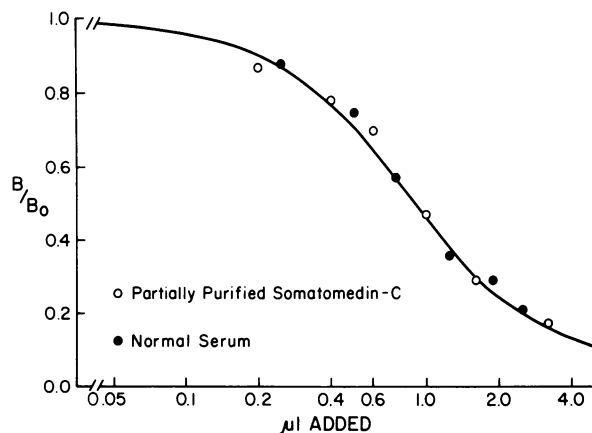


FIGURE 2 Competitive-dose response curves for the serum standard (●) and partially purified somatomedin-C (○) by the nonequilibrium assay procedure. Each test sample was incubated with antiserum for 72 h before  $^{125}\text{I}$ -somatomedin-C was added. The partially purified preparation of somatomedin-C had a specific activity of 116.2 U/mg. The standard solution used contained 8.6  $\mu\text{g/ml}$ .

thyrotropin and ovine follitropin inhibit  $^{125}\text{I}$ -somatomedin-C binding only at concentrations  $> 1 \mu\text{M}$ . Porcine insulin, which cross reacts in the somatomedin-C placental membrane receptor assay, is also recognized by the somatomedin-C antibody but only at very high concentrations. Moreover, the competitive dose-response curve for insulin is not parallel to the somatomedin standard (Fig. 3). Highly purified somatomedin-A produces a competitive dose-response curve parallel to that of somatomedin-C, but it is only 3% as active as somatomedin-C on a weight basis.  $^{125}\text{I}$ -MSA (multiplication-stimulating activity obtained from cultures of cloned rat liver cells) failed to bind to the antiserum under the conditions employed in the assay. Purified nonsuppressible insulin-like activity was not available for comparison.

Further evidence for the specificity of the somatomedin-C antibody was obtained during the course of purifying somatomedin from extracts of Cohn fraction IV of freshly frozen human plasma. After isoelectric focusing most of the activity measured by the radioimmunoassay was detected in the basic fractions, whereas a substantial quantity of activity with a neutral isoelectric point was additionally measured by competitive cell membrane receptor assays using either  $^{125}\text{I}$ -somatomedin-C or  $^{125}\text{I}$ -insulin as the radioligand. These studies will be the subject of a subsequent publication.<sup>5</sup>

A number of chemically modified derivatives of somatomedin-C were tested for possible immuno-

<sup>5</sup> Van Wyk, J. J., C. J. Decedue, and R. J. Schlueter. To be published.

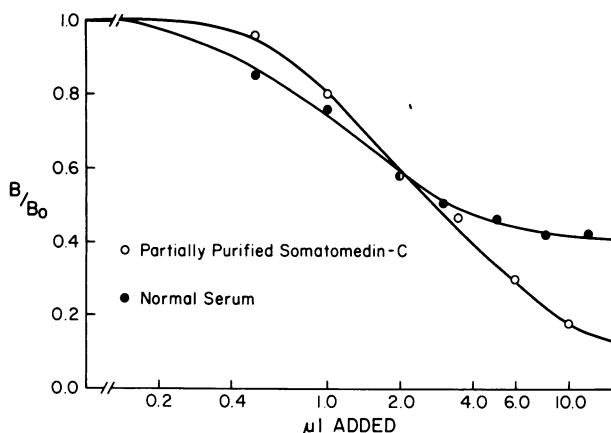


FIGURE 1 Competitive dose-response curves for the serum standard (●) and partially purified somatomedin-C (○) by the equilibrium assay procedure. Each test sample was mixed with  $^{125}\text{I}$ -somatomedin-C and the reaction was initiated by the addition of antiserum. The partially purified preparation of somatomedin-C had a specific activity of 116.2 U/mg and the standard solution used contained 6.9  $\mu\text{g/ml}$ .

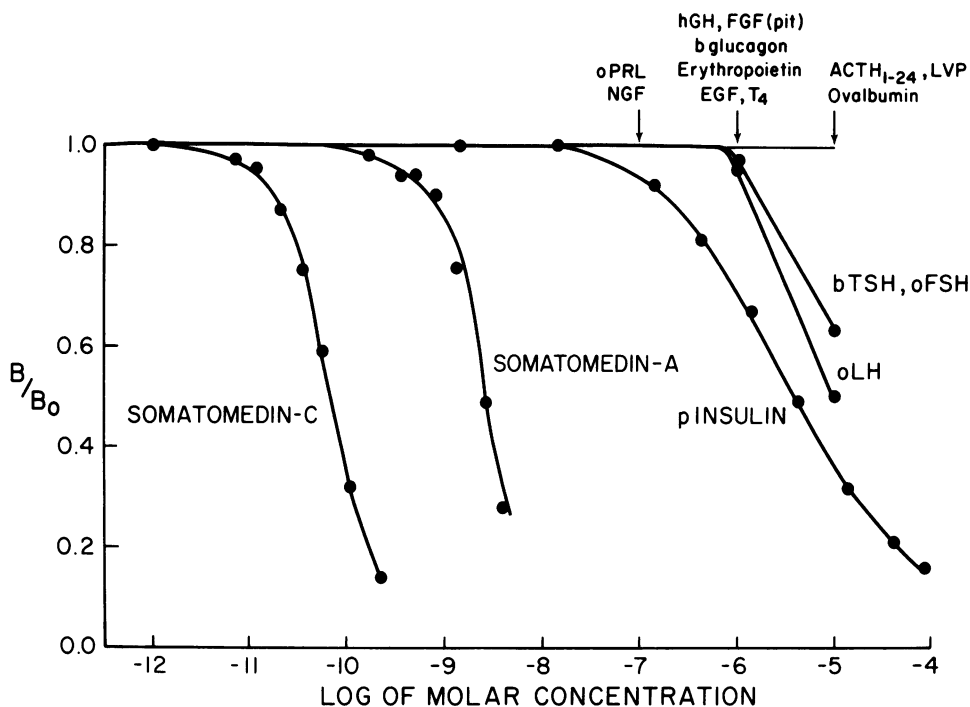


FIGURE 3 Hormonal specificity of the somatomedin-C antiserum. The assays were performed by the nonequilibrium assay procedure. Molar concentrations were calculated assuming a molecular weight of 6,000 for both somatomedin-A and somatomedin-C. ACTH<sub>1-24</sub>, N-terminal tetraeicosapeptide of corticotropin; EGF, mouse epidermal growth factor; FGF (pit), pituitary fibroblast growth factor; oFSH, ovine follitropin; oLH, ovine lutropin; NGF, nerve growth factor; oPRL, ovine prolactin; hGH, human somatotropin; bTSH, bovine thyrotropin; T<sub>4</sub>, thyroxine; LVP, lysine vasopressin.

logic activity. These derivatives included performic acid-oxidized somatomedin-C, reduced and carboxamidomethylated somatomedin-C, and the fragments obtained by cyanogen bromide treatment and trypsinization of the reduced, carboxamidomethylated material. The preparation of these derivatives is described elsewhere (16a). All derivatives retained <1% of their original immunologic activity, suggesting that the antibody has a high degree of specificity for the intact somatomedin structure.

*Nature of somatomedin-C in serum.* After gel filtration at neutral pH on Sephadex G-200, 95% of the somatomedin activity in serum is detected in a peak corresponding to the immunoglobulins or slightly smaller molecules (Fig. 4). The remaining 5% of the activity is found in a region corresponding to a molecular weight of approximately 35,000. The association of somatomedin activity in sera with large molecular weight proteins is believed to be due to the presence of specific binding proteins for somatomedin (17, 18, 18a).

The elution pattern for <sup>125</sup>I-somatomedin-C, which had been preincubated with serum before chromatography, is quite different from that of endogenous

somatomedin-C. As shown in Fig. 4, radioactivity is distributed into three main peaks: the first peak corresponds with the primary endogenous peak, a second, much larger peak corresponds with the small secondary endogenous zone, and a third peak corresponds to free somatomedin-C.

*Gel filtration of serum and of partially purified somatomedin-C in 1 M acetic acid.* The effects of the somatomedin-C binding proteins on this radioimmunoassay were further evaluated by comparing the somatomedin concentrations in unextracted serum with those found after somatomedin had been dissociated and separated from its binding protein. This was accomplished by gel filtration of serum on Sephadex G-50 in 1 M acetic acid. Under these conditions somatomedin has a  $K_{av}$  of approximately 0.4 and is clearly separated from the majority of serum proteins. Recoveries of immunoassayable somatomedin, relative to the activities in the unextracted sera, were 116, 128, and 94%, respectively, for acromegalic, normal, and hypopituitary sera. Under similar conditions, the recovery of partially purified somatomedin was 110%. For all samples, approximately 95% of the activity was found in the region between  $K_{av}$  0.2 and 0.8. These experi-

ments establish that somatomedin-C can be quantitatively measured in native serum by the nonequilibrium technique without the need for prior chemical dissociation from the binding protein.

**Somatomedin-C activity in human serum.** Somatomedin-C concentrations in serum were measured by the nonequilibrium technique using sample volumes of 0.02–10  $\mu$ l/tube. Relative to our standard pool of serum from three normal adult males (arbitrarily assigned a value of 1 U/ml), somatomedin-C concentrations in normal subjects were lowest in cord sera ( $0.38 \pm 0.05$  U/ml [SEM]) and rose dramatically during early childhood to reach concentrations of  $1.30 \pm 0.16$  U/ml [SEM] by 4 yr of age (Fig. 5). The 23 normal adult subjects had a mean serum somatomedin-C concentration of  $1.50 \pm 0.10$  U/ml (SEM). Although the supply of pure somatomedin-C was too limited to permit its use as a primary standard in routine assays, indirect comparisons suggest that the concentrations of somatomedin-C in the serum of normal adults is in the range of 75–200 ng/ml.

To determine whether the substance measured by the radioimmunoassay is growth hormone dependent, the serum somatomedin-C concentrations in normal subjects were compared to those of children with hypopituitary dwarfism and adults with acromegaly. The

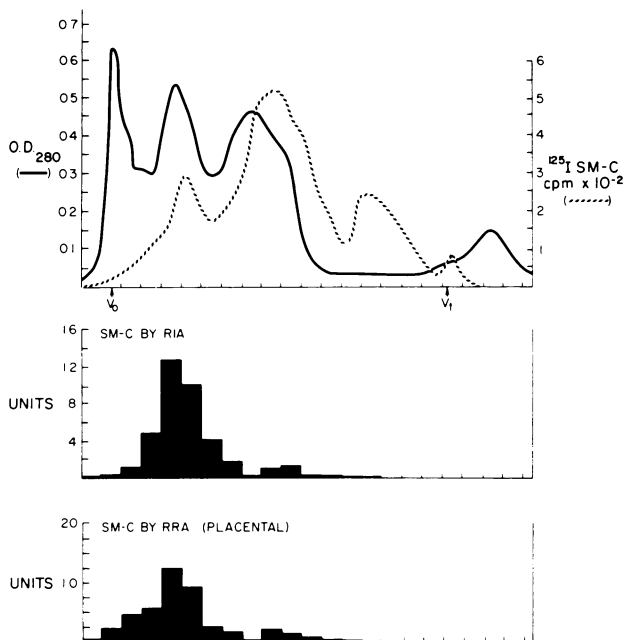


FIGURE 4 Gel filtration of normal serum containing  $^{125}\text{I}$ -somatomedin-C (approximately 300,000 cpm; 15 fmol) on Sephadex G-200 at pH 7.6. Upper panel: (—) OD at 280 nm, (· · ·) cpm of  $^{125}\text{I}$ -somatomedin-C. Center panel: somatomedin-C activity of each fraction determined by radioimmunoassay. Lower panel: somatomedin-C activity as determined by the placental membrane binding assay.

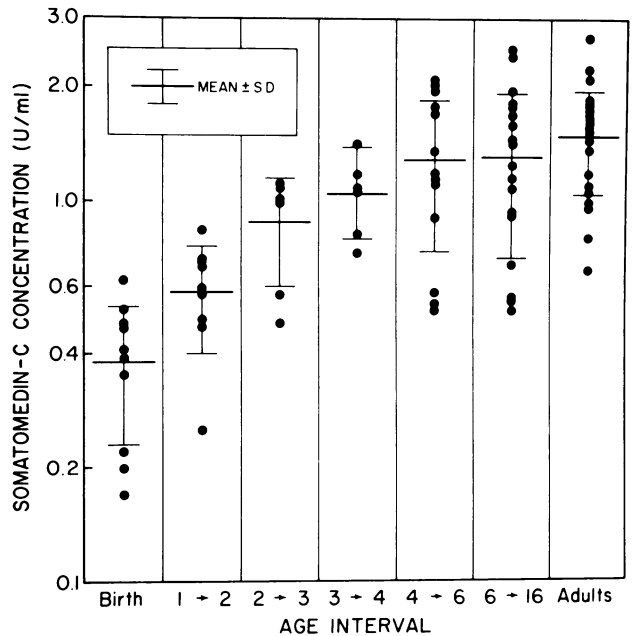


FIGURE 5 Serum somatomedin-C concentrations in relation to age for 68 normal infants and children. Age intervals are in years. Assays were performed by the nonequilibrium assay procedure. Bars indicated the mean  $\pm$  1 SD.

14 active acromegalics had a mean concentration of  $6.28 \pm 0.37$  U/ml (SEM); ( $P < 0.0005$  by the student's  $t$  test). The 19 hypopituitary children all had levels below 0.2 U/ml. 17 of these had levels below 0.1 U/ml, the lower limit of assay sensitivity (Fig. 6). The difference between the normal children (ages 5–16) and the hypopituitary children is highly significant ( $P < 0.0005$  by the  $t$  test). Further evidence for the growth hormone dependency of the substance measured by the assay is provided by the studies of somatomedin induction after the administration of growth hormone (Fig. 7).

The serum somatomedin-C concentrations in three patients with Cushing's disease were moderately elevated ( $2.61 \pm 0.14$  U/ml [SEM];  $P < 0.005$  by the  $t$  test), whereas in three patients with hyperprolactinemia the concentrations were normal ( $1.74 \pm 0.11$  U/ml [SEM]).

**Somatomedin in other species.** Somatomedin-C activity can be quantitated in the sera of several sub-human species (Fig. 8). Only the competitive binding curve for rat serum was not parallel to the human serum standard. Dog sera were approximately equipotent with human serum. Guinea pig and baboon sera were about three times as potent as human serum, whereas lamb and chicken sera were only 1/20 as active. Only Atlantic blue fish serum was found to be totally devoid of activity.

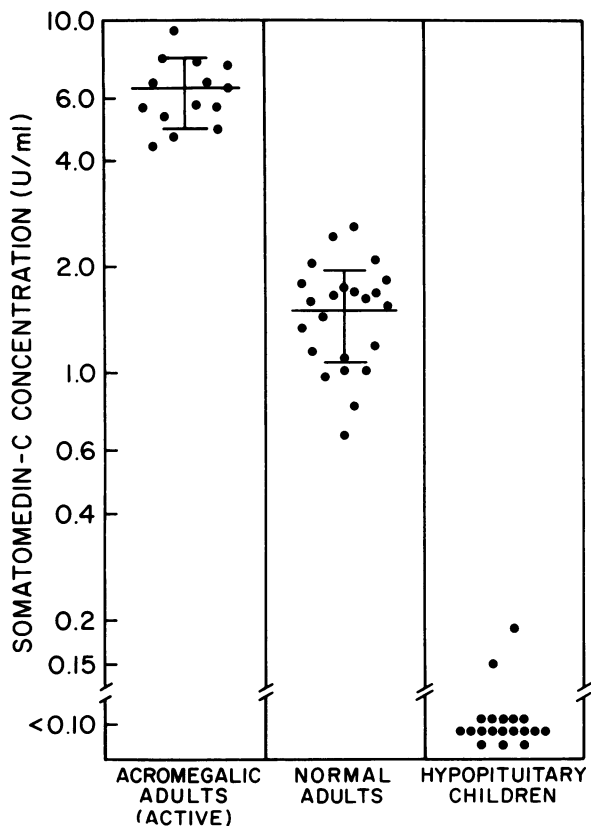


FIGURE 6 Serum somatomedin-C concentrations in acromegalic adults, normal adults, and untreated hypopituitary children. Assays were performed by the nonequilibrium assay procedure. Bars indicate the mean  $\pm$  1 SD.

### DISCUSSION

In initial experiments, the usual "equilibrium" assay method was utilized. This entails the simultaneous addition of trace, antibody, and standard or unknown sample. Under these conditions, the competitive dose-response curves for serum and for partially purified hormone were not parallel, and, at high serum concentrations, the curve plateaued at a higher  $B/B_0$  value than observed with partially purified hormone. A possible explanation for these findings comes from the work of Rodbard et al. (13) who showed that before attaining equilibrium, the shape of the dose-response curve for any competitive protein binding assay system is dependent on the order of the addition of reactants. With computer-simulated models in which the order of addition of reactants was varied, they found that under the classic assay conditions, equilibrium is reached most rapidly at high concentrations of added antigen. In contrast, under the unusual condition where antibody is preincubated with radio-labeled hormone before the addition of unlabeled antigen, the time required to reach equilibrium is pro-

longed and equilibrium is reached more slowly at high concentrations of added antigen. As a result, at any time before achieving equilibrium, the slope of the dose-response curve is diminished, and the  $B/B_0$  values are elevated at high concentrations of antigen.

These considerations could apply to the somatomedin-C radioimmunoassay if free somatomedin (either labeled or unlabeled) reacts with the antibody more rapidly than does endogenous hormone. Such circumstances would, in effect, be equivalent to preincubation of antibody and trace with the delayed addition of unlabeled antigen. The experiment shown in Fig. 4 suggests a mechanism by which this might occur. Here it was shown that most endogenous somatomedin exists in serum in a somatomedin-binding protein complex which is not readily available for reaction with antibody. Because  $^{125}\text{I}$ -somatomedin, when added to serum, does not rapidly equilibrate with the complex, it is more readily available to react with antibody when the equilibrium assay method is employed.

This hypothesis can be verified only by determin-

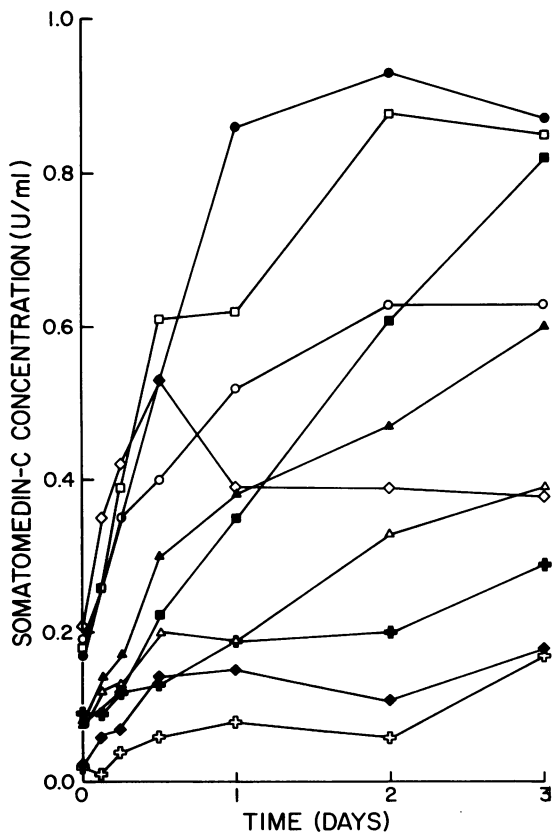


FIGURE 7 The induction of immunoreactive somatomedin-C in 10 hypopituitary children after the intravenous and intramuscular administration of human growth hormone.



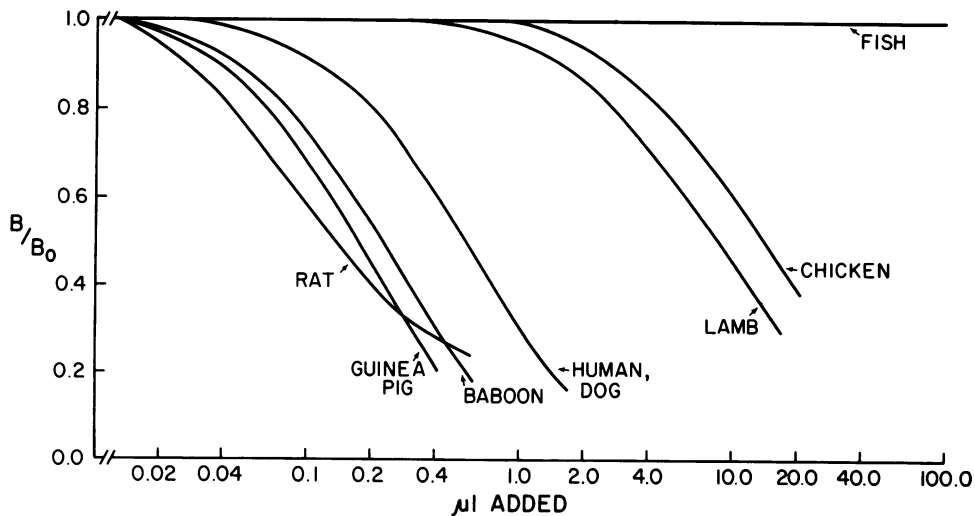


FIGURE 8 Immunoreactive somatomedin-C concentrations in the sera of various animal species. The curve for human serum was determined with the somatomedin reference standard (MUC), but was adjusted to the mean activity of normal adult volunteers (MUC activity  $\times 1.5$ ). The fish serum was obtained from the Atlantic bluefish (*Pomatomus saltaris*).

ing the appropriate rate constants of the components of the reaction. The model, however, provides a plausible explanation for the mechanism by which preincubation of samples with antibody followed by the delayed addition of trace results in parallel dose-response curves for serum and partially purified hormone. Under these conditions, the labeled hormone is used only to measure the fraction of unreacted antibody remaining after the first equilibrium reaction. Its rate of reaction relative to the rate of reaction of bound somatomedin no longer influences the shape of the dose-response curve.

When measuring hormonal concentrations in unextracted serum by any radioligand technique, it is an absolute requirement that parallelism be achieved between serum and free hormone. This does not assure, however, that the hormone is being quantitatively measured. Evidence that all of the somatomedin-C in unextracted serum is being measured by our present technique was obtained by comparing somatomedin concentrations in unextracted sera with the levels after removal of the binding protein by gel filtration under acidic conditions. The results obtained suggest that under the conditions of this assay (antibody concentration 50 pM;  $K_a$   $4.6 \times 10^{10}$  liters/mol), the antibody can effectively compete with the somatomedin binding protein.

The results reported here suggest that the antiserum has a high degree of specificity. None of the classic hormones tested, including insulin, and none of the known growth factors cross reacts at or near physiologic concentrations. It remains to be established whether the 3% cross-reactivity found in the

preparation of somatomedin-A available to us is inherent in the molecule itself or is the result of a small amount of contamination with somatomedin-C. The failure of  $^{125}\text{I}$  multiplication stimulating activity obtained from cloned rat liver cells to bind to the antibody also attests to the specificity of the antiserum. Inasmuch as a purified preparation of non-suppressible insulinlike activity was not tested, it is possible that this hormone is measured in addition to somatomedin-C.

The finding of elevated somatomedin-C concentrations in acromegaly and low concentrations in hypopituitarism provide strong evidence for the growth hormone dependency of the substance being measured. The prompt rise in serum immunoreactive somatomedin-C concentrations in 8 of 10 hypopituitary patients treated with growth hormone is further evidence for its growth hormone dependency. In the two patients who failed to exhibit a convincing increase during the initial treatment period, somatomedin-C concentrations rose to  $>0.30$  U/ml after more prolonged growth hormone therapy. The wide variation in the magnitude of somatomedin responses to growth hormone treatment has been observed by others with both bioassay and membrane receptor assay (14, 19). Some degree of growth hormone priming may be necessary for adequate somatomedin responses because patients with very low basal immunoreactive somatomedin-C concentrations tended to have only small increments after growth hormone treatment.

The low levels of somatomedin-C observed in cord blood and the striking increase in concentration in the first few years of life were nearly identical to the

results obtained by bioassay (20) and by the somatomedin competitive membrane binding assay (14). The explanation for the relatively low somatomedin-C levels in early life is not apparent.

With the sulfation factor bioassay, no clear picture of the effects of cortisol on somatomedin action have emerged. States of cortisol excess have been reported to result in both diminished (20–22) and moderately elevated serum somatomedin activity (23). Furthermore, in vitro addition of microgram quantities of glucocorticoids to cartilage incubates has been shown to diminish sulfate uptake (24) or to have no significant effect (25). Although our results must be confirmed by more extensive studies, the finding that under conditions of cortisol excess immunoreactive somatomedin-C concentrations are, in fact, modestly elevated suggests that the inhibitory effect of cortisol observed in sulfation factor assays is at the level of chondrocyte and not at the level of somatomedin synthesis.

The most striking feature of the radioimmunoassay results is that the magnitude of the differences in immunoreactive somatomedin-C in acromegalic, normal, and hypopituitary patients is much greater than those observed with other assay techniques. It is particularly noteworthy that 17 of 19 untreated hypopituitary patients have somatomedin-C concentrations of <0.1 U/ml, which is <8% of the mean value of age-matched controls. In contrast, with the exception of the hypophysectomized rat costal cartilage assay as performed by Daughaday (26), in most bioassays and somatomedin membrane binding assays, hypopituitary serum has been observed to have 30–40% of the activity found in normal control serum (14, 20, 26–28). In addition, when the somatomedin concentration in the hypopituitary children was measured by the competitive membrane binding assay on the same serum samples used in the radioimmunoassay, the mean somatomedin concentration was 33% of the age-matched normal children. These results suggest that the somatomedin-C radioimmunoassay is more specific than either bioassays or competitive membrane binding assays. In addition to detecting somatomedin-C, the latter assays detect other substances in serum which apparently are not under as stringent growth hormone control.

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