Purification of Somatomedin-C from Human Plasma: Chemical and Biological Properties, Partial Sequence Analysis, and Relationship to Other Somatomedins†

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ABSTRACT: Somatomedin-C (SM-C) was purified from Cohn fraction IV_{1-4} of human plasma by a series of steps which included cation-exchange chromatography, gel filtration, isoelectric focusing, and reverse-phase high-pressure liquid chromatography. The peptide isolated contained 10 142 units/mg, as judged by a radioimmunoassay, representing a 780 000-fold purification from native plasma. The isolated peptide was basic (p*I* 8.1–8.5) and was judged to be no less than 90% pure. The best fit for integral amino acids was obtained with 78 residues. Limited sequence data were obtained on the N terminus and on five fragments obtained by tryptic digestion after blocking the lysine residues. Three of these fragments and the N terminus could be aligned with portions of insulin-like growth factor I (IGF-I). Of the 25 residues so aligned, 22 were identical with IGF-I. In addition, two tryptic fragments were obtained which are not present in the sequence of IGF-I. SM-C and IGF-I produced identical curves of displacement in radioimmunoassay and radioreceptor assays for SM-C and in an insulin radioreceptor assay. The potencies of SM-C and IGF-I in these assays differed significantly from IGF-II, somatomedin-A, and several preparations of multiplication stimulating activity. At a concentration of 1 ng/mL, SM-C stimulated the progression of Balb/c 3T3 cells into DNA synthesis and when injected in vivo restored mitosis in lens epithelium of hypophysectomized frogs.

It has been postulated that the somatic growth which follows growth hormone adminstration in vivo is mediated through a family of mitogenic, insulin-like peptides whose serum concentrations are growth hormone dependent (Daughaday et al., 1972; Van Wyk & Underwood, 1978). Somatomedin-C (SM-C[1]) is the designation for a basic peptide (p*I* 8.0–8.7) of ~7500 daltons which was partially purified from human plasma on the basis of its capacity to stimulate $^{35}SO_4$ and [^{3}H]thymidine incorporation into cartilage explants from hypophysectomized rats (Van Wyk et al., 1974, 1975). Other human somatomedins include somatomedin-A (SM-A), a neutral peptide which was likewise purified on the basis of a cartilage assay (Hall, 1972; Fryklund et al., 1974), and two distinct insulin-like growth factors (IGF-I and IGF-II) which were purified on the basis of their insulin-like activities in rat adipose tissue (Rinderknecht & Humbel, 1976a,b). Several similar peptides known as "multiplication stimulating activity" (MSA) have been purified from rat liver culture medium on the basis of their mitogenic activity for chick embryo fibroblasts (Dulak & Temin, 1973; Nissley & Rechler, 1978). Amino acid sequences have been reported only for IGF-I and IGF-II (Rinderknecht & Humbel, 1978a,b).

This paper will describe an improved procedure for the isolation of SM-C from Cohn fraction IV of human plasma by using a radioimmunoassay for SM-C to monitor purification. The isolated peptide was found to be a potent mitogen and was identical with IGF-I in three radioligand assays, each based on different properties of the molecule. Although the amino acid sequence of SM-C remains incomplete because of insufficient material, several but not all tryptic fragments were found to be identical with corresponding residues in IGF-I.

Materials and Methods

Cohn fraction IV_{1-4} paste prepared from large pools of fresh frozen plasma was stored at −10 °C until ready for use.[2] Thirty grams of this paste was equivalent to 1 L of whole plasma. Sephadex and SP-Sephadex were from Pharmacia. Ampholine and ampholine–polyacrylamide PAGplates were from LKB. Solvents for LC were from Fisher Scientific, and highly purified acetic acid was from G. Frederick Smith Chemical Co. Na^{125}I was from Amersham, and [^{125}I]insulin was from Cambridge Nuclear. [^{125}I]Somatomedin-C (~400 μCi/μg) was prepared by a fractional chloramine-T method. The iodinated peptide was purified by affinity chromatography using the IgG fraction of a rabbit antihuman somatomedin-C antiserum coupled with Sepharose 4B.

Radioligand Assays. Radioreceptor assays for SM-C and insulin were carried out as previously described by Marshall et al. (1974), and the radioimmunoassay (RIA) for SM-C was carried out as described by Furlanetto et al. (1977). One unit of SM-C is defined as the SM-C content, as determined by RIA, in 1 mL of a serum pool obtained from over 1000 normal blood donors (Ortho Control Serum, lot no. 1777-2).

The insulin standard, lot no. 818194 containing 24.8 units/mg, was a gift from Dr. Mary Root, Eli Lilly Co. Insu-

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[1] Abbreviations used: BAWP, butanol–acetic acid–water–pyridine; DMAA, dimethylallylamine; LC, high-pressure liquid chromatography; IGF, insulin-like growth factor; MSA, multiplication stimulating activity; PTH, phenylthiohydantoin; RIA, radioimmunoassay, RRA, radioreceptor assay; $NaDodSO_4$, sodium dodecyl sulfate; SM-A, somatomedin-A; SM-C, somatomedin-C; TLC, thin-layer chromatography.

[2] We are indebted to Dr. Joseph A. Daley, Director of Research of the Armour Pharmaceutical Co., Kankakee, IL, for providing generous quantities of Cohn fraction IV_{1-4}.

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lin-like growth factors I and II were gifts from Drs. Rene Humbel and Jürgen Zapf of the University of Zurich. Somatomedin-A (SM-A) was a gift from Drs. Linda Fryklund and Hans Sievertsson of AB Kabi, Stockholm, Sweden, and the preparations of multiplication stimulating activity (MSA II-1 and MSA III-2) were gifts from Dr. Matthew Rechler of NIAMDD, Bethesda, MD. Each of the above preparations was considered by their donors to be homogeneous by several criteria.

Proteins were determined by the optical density method of Waddell (1956) or by the method of Lowry et al. (1951) using bovine albumin as the standard. The final quantitation of protein was by summation of amino acids in acid hydrolysates using a Beckman Model 121 M amino acid analyzer. The UV spectrum of the purified peptide was obtained with a Cary Model 15 recording spectophotometer.

Cell Growth Assays. Assessment of biological activity was made by the progression factor assay as described by Stiles et al. (1979). In this method, confluent Balb/c 3T3 cells are pulsed with platelet growth factor for 5 h, after which the test preparation is incubated for an additional 24 h in the presence of [^{3}H]thymidine and 5% platelet poor plasma from a patient with severe hypopituitarism. After 24 h in the test solution, the cells are fixed, developed with a photosensitive emulsion, and counterstained with Giemsa. Progression factor activity is manifested by the increase in nuclear labeling over that observed in the presence of hypopituitary serum alone.

Purification of Somatomedin-C. Most of the studies on the pure peptide reported in this paper were carried out on a single batch of Cohn IV paste purified as described below.

Extraction. Thawed Cohn IV_{1-4} paste, 3 kg, was homogenized in an industrial Waring blender with 30 L of 2.0 M acetic acid and 0.075 M NaCl. A large amount of insoluble protein was removed in a Sharples centrifuge. The cloudy extract, which contained large quantities of lipoproteins, had a pH of 2.84 and a conductivity of 7 mmhos.

Chromatography on SP-Sephadex and Sephadex G-50. The extract was passed over a 5 × 35 cm cationic exchange column containing SP-Sephadex C-25 which had been equilibrated to the pH and conductivity of the extract. For prevention of clogging of this column with lipoproteins and residual suspended proteins, a smaller renewable column of the same resin was installed before the large column to serve as a trap. After the extract had all been passed through the columns, the combined resins were washed extensively with starting buffer. Elution was accomplished at 7 mL/min with 1.5 M NaCl, 1 M in ammonium acetate, pH 7. When the column was stripped with the last buffer titrated to pH 9.0 with aqueous ammonia, a small ammount of residual SM-C was eluted. All fractions containing SM-C activity were pooled and dialyzed in 54-mm Spectrapore III tubing (Spectrum Industries) against running tap water for 18 h. The retentate was adjusted to starting buffer conditions, applied at 3 mL/min to an SP-Sephadex C-25 column (2.5 × 30 cm), washed with 550 mL of starting buffer, and eluted with 1650 mL of starting buffer containing 0.2 M NaCl and then with 1650 mL of this buffer containing 0.4 M NaCl and finally by using a pH step gradient (750 mL each) at pH values of 5.0, 6.0, and 9.0. Each of these buffers contained 0.2 M ammonium acetate and 0.2 M NaCl and was titrated with aqueous ammonia to the desired pH. The fractions containing SM-C activity were pooled, dialyzed, and lyophilized.

The dried product was dissolved in 180 mL of 1 M acetic acid and chromatographed on a 10 × 94 cm column of Sephadex G-50 in 1 M acetic acid. Fractions with SM-C activity were pooled, adjusted to a pH of 2.84 and a conductivity of 7 mmhos, applied at 3 mL/min to a third column of SP-Sephadex C-25 (2.5 × 15 cm), and washed with starting buffer and then with buffers at pH 4.0 and 4.5 continuing in each case until the absorbance returned to base line. Elution was achieved with a linear salt gradient (390 mL) at pH 4.5 from 0.2 M NaCl to a limit concentration of 2 M NaCl. Again, the SM-C-containing fractions were pooled, dialyzed, and lyophilized. The dried product in 25 mL of 1 M acetic acid was chromatographed on a 5 × 90 cm Sephadex G-50 column in 1 M acetic acid. The SM-C-containing fractions were pooled and lyophilized.

Flatbed Isofocusing. A slurry of 35 g of prewashed Sephadex G-75 SF in 750–800 mL of an aqueous solution of the protein containing 30 mL of ampholine, pH 5–10, was poured into a 20 × 40 cm flatbed tray (Brinkman Industries) and air-dired to proper consistency. Platinum ribbon electrodes were placed on paper strips, soaked in 1 M phosphoric acid at the anode and 1 M NaOH at the cathode. Cytochrome *c* solution was streaked across the plate near the anode to serve as a marker. Isofocusing was carried out in a Brinkman water-cooled isofocusing chamber for ~36 h while the voltage was increased from 100 to 800 V. The isofocusing was terminated when the cytochrome *c* had reached the cathode and the amperage was stable. Immediately after completion, 1-cm sections were removed to beakers with 40 mL of water and the pH and OD_{280} were determined. SM-C-containing fractions with a pH greater than 7.8 were eluted from the Sephadex and lyophilized. The previous isoelectric focusing procedure was then repeated using ampholines pH 7–9.5. Those fractions which displayed SM-C activity in the pH range 8.1–8.5 were eluted and chromatographed on a 1.9 cm × 2 m Sephadex G-50 F column in 1 M acetic acid. Fractions of 15 mL were collected in acid-washed siliconized tubes. Those fractions containing SM-C were pooled and lyophilized. The chromatographic procedure was repeated.

LC. All solvents and buffers used in LC were first passed through a 0.2-μm Millipore membrane. Only glass-distilled deionized water was used. Reverse-phase LC was carried out by using a Waters Associates Model 6000A solvent delivery system equipped with a Waters U6K injector and a 7.8 mm × 30 cm μBondapak alkylphenyl semipreparative column. Aliquots of the protein solution were injected with a Hamilton gas-tight syringe and eluted in a stepwise gradient of acetonitrile in 0.01 M KH_2PO_4. Elution at 5 mL/min was begun with 25% acetonitrile for 20 min, followed by 15 min with 28.8% acetonitrile, 15 min with 30% acetonitrile, and finally with 40% acetonitrile until base line was reached. The OD at 280 nm was measured with an ISCO Model UA5 monitor with 1-cm "Z" cells in the optical unit. One-minute (5-mL) fractions were collected in siliconized tubes.

Those fractions corresonding to the major peak of SM-C activity were pooled, lyophilized, dissolved in 10 mL of 1 M acetic acid, and chromatographed on a Sephadex G-50 F column (1.9 × 200 cm) in 1 M acetic acid to effect desalting. LC side fractions containing lesser amounts of activity were also pooled and rechromatographed in the same system by using a 3.9 mm × 30 cm analytical μBondapak alkylphenyl column. The SM-C-containing fractions were processed as above.

Dansylation. The dansylation of 8.75 μg of SM-C was carried out as described by Tamura et al. (1973) using procedure B. The lyophilized dansylated product was dissolved in 20 μL of methanol and applied to a silica gel TLC plate (Anasil H, Analabs, Inc.). The plate was first developed in

acetone, dried, and then developed in butanol–acetic acid–water–pyridine (BAWP) (15:3:12:10). The fluorescent spots were scraped off the plate and eluted with acetone–acetic acid–water–pyridine (50:1:50:3) followed by elution with BAWP (15:3:12:10). The eluates were dried by rotary evaporation and assayed for SM-C.

$NaDodSO_4$–Polyacrylamide Gel Electrophoresis. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of the purified SM-C and reference proteins was carried out in a Bio-Rad Model 220 vertical slab gel cell according to the method of Laemmli (1970) with the exception that 2-mercaptoethanol was omitted. Ten micrograms of SM-C and the reference proteins were dissolved in 35 μL of a solution containing 1% $NaDodSO_4$ and 0.04 M K_2HPO_4 at pH 8.2 and heated at 37 °C for 2 h. Five microliters of Fluoram (Roche), 20 μg/mL in acetone, was added with vigorous mixing followed by the addition of 10 μL of 80% glycerol containing bromphenol blue. The entire solution of each protein was applied to the wells of the gel and was run at 20 mA for 135 min followed by 30 mA for 1 h.

The gel was removed and photographed by using long wavelength UV light as the excitation source. The portion of the SM-C gel which displayed fluorescence was cut into 10 equivalent horizontal slices and the protein eluted by the process described by Rinderknecht & Humbel (1976a). The eluted peptide was lyophilized and the SM-C content determined.

Polyacrylamide gel electrophoresis at pH 4.3 was carried out according to Maurer (1971) with the exception that the running gel was 18% and the concentrating gel 3%. Proteins from 8 to 15 μg were dissolved in 100 μL of water and 100 μL of a 1:10 upper electrode buffer containing 10% glycerol and the tracking dye pyranine Y. After electrophoresis, the gel containing the SM-C preparation was sliced longitudinally. One-half was fixed in trichloroacetic acid (Cl_3AcOH) and stained with Coomassie Brilliant Blue R250 (Bio-Rad) as were the other gels. The other half was horizontally sliced into 40 sections, 1.3 mm in width, which were allowed to stand in 1 mL of 1 M acetic acid for 4 days and were lyophilized. One milliliter of water was added to each tube, and an aliquot was assayed for activity by the SM-C RIA.

Analytical polyacrylamide–ampholine isofocusing was carried out by using PAGplates from LKB according to the manufacturer's directions. The plates were fixed and stained with Coomassie Blue R250.

Amino Acid Analysis. Amino acid analyses were performed in quadruplicate on samples which were hydrolyzed at reduced pressure at 112 °C in 6 M constant boiling HCl for 24, 48, and 72 h. Norleucine, 16 nM, was added to the orginal sample as an internal standard to correct for recoveries. Cysteine was determined as cysteic acid after performic acid oxidation at room temperature for 1 h followed by hydrolysis in 6 HCl at reduced pressure for 24 h. Hydrolysates were chromatographed on a Beckman 121 M amino acid analyzer equipped with an Autolab automatic integrator to calculate the quantity of each amino acid. The average nanomolar content at the three time periods was used for all amino acids except Thr, Met, and Ser, where the values were extrapolated to zero time. These concentrations were entered into a computer program which determined the number of residues of each amino acid in the intact molecule either as a function of variable numbers of total residues or by assigning fixed numbers of residues to specific amino acids.

Sequence Analysis. For determination of the N-terminal sequence, somatomedin-C was either sequenced directly or first reduced with 2-mercaptoethanol, alkylated with [^{3}H]iodoacetic acid, and then reacted with Braunitzer Reagent III (Braunitzer et al., 1971). An Edman degradation was carried out on a Beckman 890-C sequenator using the phenyl [^{35}S]isothiocyanate method described by Jacobs & Niall (1975). The PTH derivatives at each step were separated by high-pressure liquid chromatography on Partisil-10 ODS (Whatman), and the radioactivity was determined both by a Berthold LC liquid scintillation flow counter and by manual counting of individual tubes to discriminate between ^{3}H and ^{35}S.

Partial tryptic digestion was carried out by the method of Klapper & Capra (1976). Purified SM-C was extensively reduced in 7 M guanidine hydrochloride with dithiothreitol (0.05 M) and alkylated with 0.11 M [^{3}H]iodoacetic acid. The reduced and alkylated peptide was then citraconylated, desalted on G-25 in 1% ammonium bicarbonate, and digested with TPCK–trypsin for 4 h at 37 °C, after which the citraconylated lysine residues were deblocked with formic acid. The lyophilized tryptic fragments were separated by paper electrophoresis at pH 2.1. The separated fragments were identified by staining a guide strip cut from the electrophoretogram representing 10% of the peptide zone, and the corresponding unstained areas were eluted with 0.5 M NH_4OH by descending chromatography.

Amino acid sequence analyses of tryptic fragments were performed with a spinning cup Beckman 890-C sequenator, using Polybrene to stabilize the fragments in the cup (Klapper et al., 1978). A DMAA program was utilized, and conversion of the sequencer product to the stable PTH derivatives was accomplished with a Sequemat P-6 autoconverter. Identification of the PTH amino acids was by LC on μBondapak C_{18} (Waters Associates, Medford, MA) and thin-layer chromatography on polyamide sheets (Summers et al., 1973) using BBOT (Fisher) as the fluor.

Results

Extraction. The initial clarified extract of 3 kg of Cohn IV_{1-4}, derived from 100 L of fresh frozen plasma, contained 63 000 SM-C units, as measured by RIA, with a specific activity of 0.063 unit/mg of protein.

Ion-Exchange Chromatography. Approximately 93% of the SM-C activity in the clarified extract was taken up by the initial SP-Sephadex. After elution, 84% of this activity was recovered (yield 54 900 units). Ninety-two percent of this activity was recovered from the second SP-Sephadex column which was eluted in a salt and pH gradient (Figure 1). The third SP-Sephadex chromatographic separation followed by gel filtration on Sephadex G-50 (Figure 2) effected the removal of more extraneous protein with the recovery to this point of 34 800 SM-C units (Table I).

Preparative Isoelectric Focusing. The first isoelectric focusing in the pH range of 5.0–9.5 segregated 82% of the SM-C activity, as determined by RIA, into a broad zone focusing above pH 8 with the remainder focusing around neutrality (Figure 3A). By the less specific SM-C radioreceptor assay, however, approximately equal quantities of cross-reacting materials were found in the pooled eluates from the basic and neutral zones (data not shown). When the pooled eluates from the fractions above pH 7.8 were refocused in a narrower pH gradient (pH 7–9.5), 74% of the applied activity was recovered in the pH range 8.2–8.5 with a second clearly differentiated peak of activity focusing at pH 9.0–9.5 (Figure 3B). This material and the neutral shoulder of the main peak have not been further characterized.

The protein content after isoelectric focusing could not be accurately determined due to interference by ampholytes;

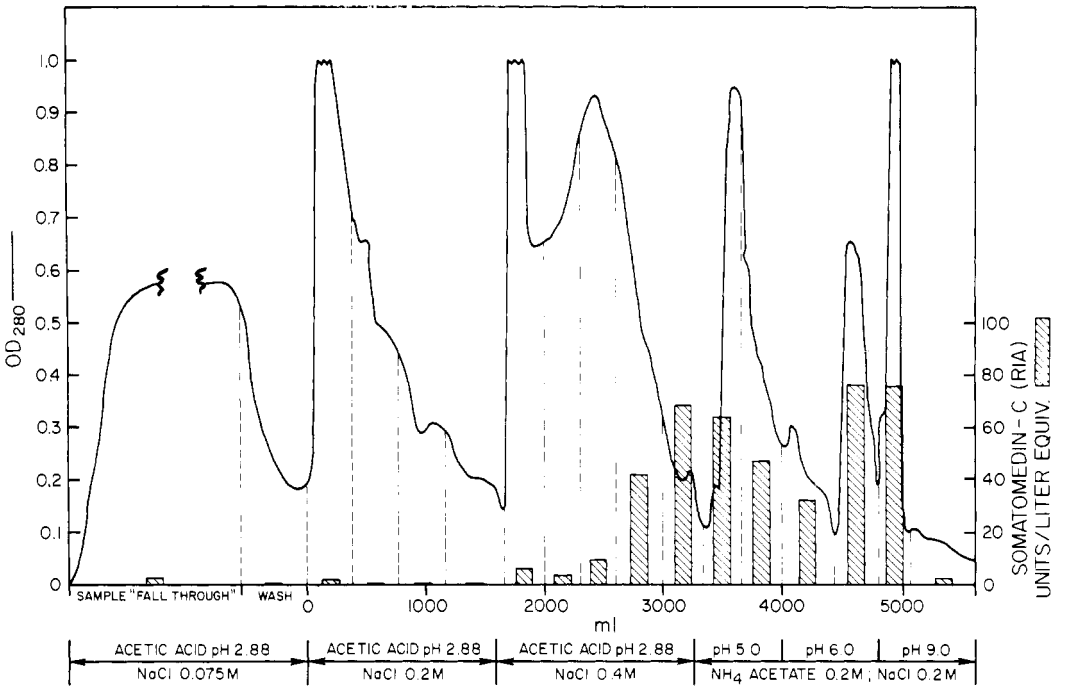

FIGURE 1: Second chromatographic separation on SP-Sephadex C-25 with elution by step gradient as indicated. The SM-C activity was determined by RIA and is here expressed as units per liter equivalent of starting plasma.

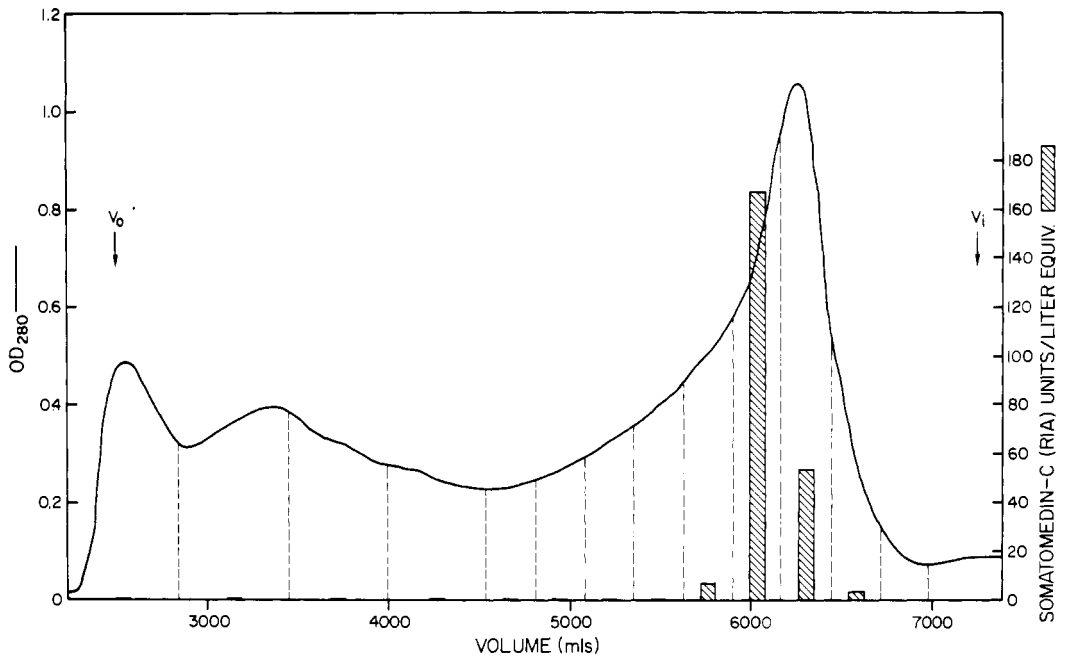

FIGURE 2: Gel chromatography on Sephadex G-50 (10 × 95 cm) in 1 M acetic acid of eluate from the third passage over SP-Sephadex. The preparation applied contained 9.8 g of protein dissolved in 180 mL of 1 M acetic acid.

however, the protein profiles observed on analytical isoelectric focusing on polyacrylamide gel revealed that prior to preparative isolectric focusing, the majority of proteins present in these extracts focused below pH 8 (Figure 4). The removal of these acidic and neutral proteins by preparative isolectric focusing accounts for most of the increase in specific activity in the subsequent two gel filtration steps after removal of the ampholytes (Table I).

LC. The LC pattern shown in Figure 5 reveals that in the solvent system used several contaminating peptides were effectively separated from the main peak of SM-C activity. The small amount of immunoreactive material which eluted in fractions preceding the main peak was resolved from the more polar extraneous proteins by rechromatography of these fractions on an analytical column.

After final desalting on Sephadex G-50 F, the protein content determined by amino acid analysis was 700 μg or 240 μg/kg of Cohn fraction IV. The overall recovery from 100 L of starting plasma was 7100 units with a specific activity of 10 142 units/mg of protein or 98 ng/unit. This represented a 780 000-fold purification from native serum. Most of the losses in the terminal steps (Table I) were accounted for by shoulder fractions since only the central area of each peak was used to achieve the final pool of purified peptide.

UV Spectrum. The UV spectrum of SM-C in water (433 μg/mL) shows the typical adsorbance maxima of phenylalanine at 247–258 nm and of tyrosine at 275 nm. The $E_{1cm}^{1\%}$ at 280 nm for this preparation is 4.2.

Table I: Major Steps in Purification of Somatomedin-C[a]

step	recovery/L of original plasma: units[b]	recovery/L of original plasma: mg of protein[c]	sp act. (units/mg of protein)	purifn (x-fold)
native plasma (theoretical)	1000	75000	0.013	0
Cohn fraction IV (clarified extract)	630	9970	0.063	4.8
first SP-Sephadex	549	ND		
second SP-Sephadex	505	98	5.15	4.0×10^2
Sephadex G-50 (after third SP-Sephadex)	348	19.5	17.8	1.4×10^3
isofocusing pH 3–10	285	ND[d]		
isofocusing pH 7–10	210	ND[d]		
Sephadex G-50 (first after isofocusing)	172	0.15[e]	1146	8.8×10^4
Sephadex G-50 (second after isofocusing	159	0.05[e]	3180	2.4×10^5
after LC and final Sephadex G-50	71	0.007	10142	7.8×10^5

[a] Starting material was 3 kg of Cohn IV paste equivalent to 100 L of plasma. [b] Recovery was determined by SM-C RIA. The standard was a commercial pool of serum from normal adults (Ortho 1777-2). [c] Proteins were determined by Lowry method. Final protein was determined by sum of amino acids in hydrolysate. [d] Proteins could not be measured due to interference by ampholytes. [e] May be spuriously high due to residual ampholytes.

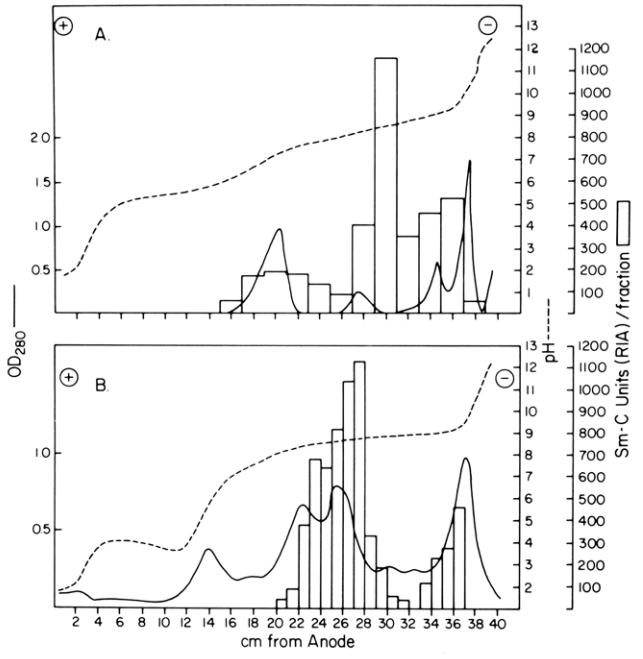

FIGURE 3: Preparative thin-layer isoelectric focusing profiles. (A) Broad range isoelectric focusing (pH 5.0–10) of the SM-C-containing fractions derived from Sephadex G-50 as shown in Figure 2. (B) Refocusing of fractions 28–32 from the first plate on a narrow range gradient of ampholines between pH 7.0 and 9.5.

Dansylation. Preliminary acetone development of the plate moved the byproducts of the dansylation process, DnsOH and $DnsNH_2$, to the solvent front, leaving the Dns peptides at the origin. After development of the plate in BAWP, fluorescence was confined to a single spot with an R_f of 0.52. A very small amount of fluorescence remained at the origin (Figure 6). The major fluorescent spot displayed activity in the SM-C RIA whereas no immunoreactive SM-C could be detected at the origin.

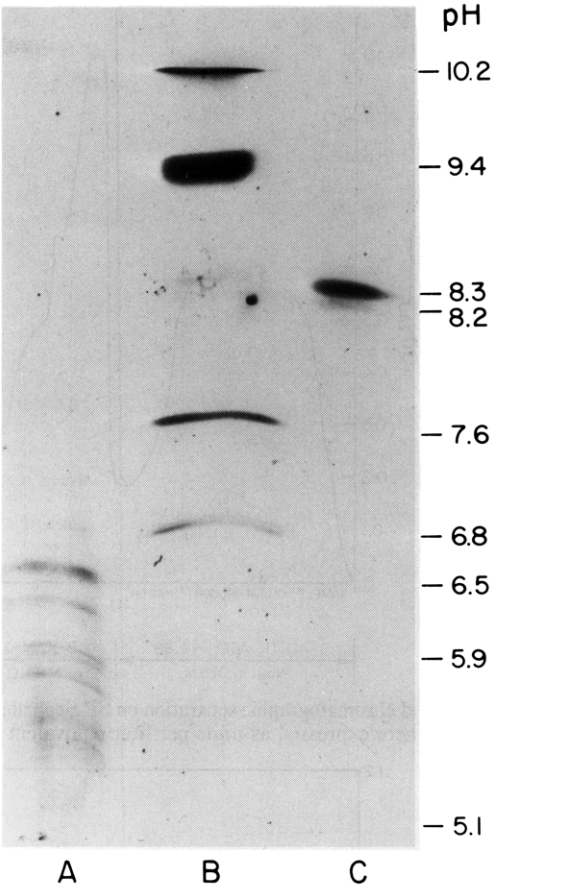

FIGURE 4: Analytical isoelectric focusing between pH 3 and 10 on LKB polyacrylamide PAGplates. Approximately 15 μg of each preparation was applied to a wick near the anode. At the completion of the run, the gels were fixed for 3 h in 30% sulfosalicylic acid and 11.5% trichloroacetic acid and then stained overnight with 0.115% Coomassie Blue R250 dye dissolved in a destaining solution of ethanol–acetic acid–water (50:16:134). (A) Pool of SM-C-containing eluate from initial SP-Sephadex step; (B) starting material for first isoelectric focusing step; (C) product isolated from second isoelectric focusing step. After LC, a comparable amount of protein failed to stain with either Coomassie Blue or Amido Black, but immunoreactive SM-C could be detected in eluates of two neighboring gel slices in the approximate region of the stained bands seen in (C).

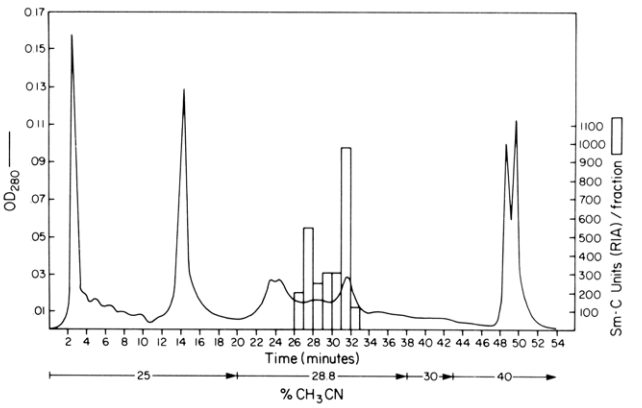

FIGURE 5: Reverse-phase LC of the product obtained after narrow range isoelectric focusing and chromatography on a Sephadex G-50 column: μBondapak alkylphenyl (7 mm × 30 cm); solvent = CH_3CN and 0.01 M KH_2PO_4; flow rate = 5 mL/min; fractions = 5 mL. Fractions eluting between 26 and 31 min were rechromatographed on an analytical column.

Polyacrylamide disc gel electrophoresis at pH 4.3 was carried out at various steps in the purification. After the initial separation on SP-Sephadex, there are many bands of slowly migrating protein (Figure 7). Following additional ion-ex-

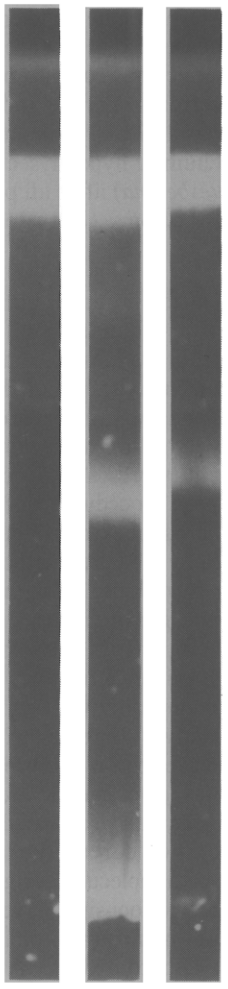

FIGURE 6: Thin-layer chromatography of dansylated peptides on silica gel. The plate was first developed in acetone to move reaction products toward the front and then, after drying, developed with BAWP (50:1:50:3). (A) Blank to identify reaction products; (B) preparation prior to LC; (C) final preparation of SM-C.

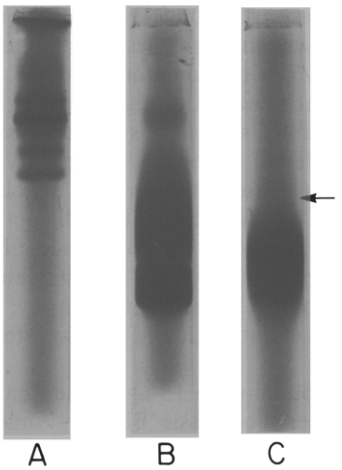

FIGURE 7: Disc gel electrophoretic pattern at pH 4.3 of preparations at various stages of purification. The running gel was 18% and staining was with Coomassie Blue R250. (A) Eluate from initial SP-Sephadex step; (B) the product prior to isoelectric focusing; (C) after isoelectric focusing. The gel containing the final product after LC did not stain, but the position of SM-C, indicated by the arrow, was identified by RIA in eluates of gel slices.

change chromatography, gel filtration, and isoelectric focusing, this extraneous protein progressively diminished and faster moving bands became apparent. After LC, no stained bands could be identified, although 8.75 μg of the peptide was applied. Assay of the elutes from the gel slices showed SM-C to be confined to two slices (2.6 mm of the 54-mm gel) which migrated with an R_f of 0.40–0.42. The R_f of the stained bands from the product prior to LC was 0.55–0.77.

Table II: Amino Acid Composition of Somatomedin-C (Residues/Mole)

	obsd[a]	nearest integer
Lys	5.04	5
His	2.05	2
Arg	5.87	6
Asp	6.00	6
Thr	3.21	3
Ser	5.97	6
Glu	6.78	7
Pro	5.06	5
Gly	6.82	7
Ala	7.64	8
1/2-Cys	3.77	4
Val	3.95	4
Met	0.92	1
Ile	1.14	1
Leu	7.07	7
Tyr	2.33	2
Phe	3.99	4
total residues		78
calcd M_r		8567

[a] Calculated by computer by assuming 6 residues of Asp per mol.

$NaDodSO_4$–Polyacrylamide Gel Electrophoresis at pH 8.8. After $NaDodSO_4$–polyacrylamide gel electrophoresis of the fluorescamine derivative of purified SM-C, most of the fluorescence was concentrated in a band which migrated with an R_f of 0.51. A faint, faster moving band, visually estimated to fluoresce with no more than 10% the intensity of the main band, was also present. SM-C immunoreactivity in eluates of gel slices was entirely confined to the major band.

The fluorescamine derivative of SM-C migrated between that of lima bean trypsin inhibitor (M_r 9000), which had an R_f of 0.49, and porcine insulin (M_r 6000), which had an R_f of 0.64. Although SM-C was closer to the 9000-dalton marker than to insulin, a more precise estimation was not possible due to nonlinearity when the log molecular weights of the entire series of marker proteins were plotted vs. their R_f values. Myoglobin (M_r 17 800) had an R_f of 0.37 and ovalbumin (M_r 45 000) an R_f of 0.19.

Amino Acid Analysis. By computer analysis, the closest approximation to integral numbers was obtained either by assigning an integral value of 6 residues to Asp or by assigning a total of 78 residues (M_r 8567). These data are shown in Table II.

Partial Sequence Analysis. Preliminary Edman degradation of two aliquots, ~10 nM each, identified glycine as the N-terminal amino acid. Six of the first nine residues were tentatively identified as G–E–L–GAE. A subsequent attempt to determine the N-terminal sequence with a larger quantity of peptide led to loss of the sample from the cup.

Approximately 30 nM was then subjected to partial tryptic digestion after blocking the lysine residues as described under Materials and Methods. Following paper electrophoresis at pH 2.1, nine zones were identified in the stained edge of the strip and the corresponding nonstained zones were eluted. Three of these zones contained insufficient peptide for further sequencing, and one zone contained an unresolved mixture. The latter fraction contained a substantial amount of radioactivity, thus suggesting the presence of one or more alkylated

Table III: Progression Factor Activity of Somatomedin-C[a]

addn to confluent Balb/c 3T3 cells			
preincubn (5 h)	incubn (24 h)		
platelet-derived growth factor pretreatment for 5 h	platelet poor plasma	somatomedin-C (ng/mL)	% nuclear labeling at 24 h
+			2
−	5% from normal donor		1
+	5% from normal donor		68
−	5% from hypopituitary donor		5
+	5% from hypopituitary donor	1	20
+	5% from hypopituitary donor	2.5	35
+	5% from hypopituitary donor	5.0	55
+	5% from hypopituitary donor	7.0	65

[a] This assay was carried out as described by Stiles et al. (1979) by Dr. W. J. Pledger, Department of Pharmacology and Cancer Research Center, University of North Carolina.

cysteine residues. Partial sequences were obtained on the other five fragments eluted from the paper. These sequences were

I: LCQLSC–E

II: DAPSTCIV––CC

III: LE––C

IV: GF–F

V: GPI–V–T–P

Activity in Radioligand Assays. At a concentration of 1400 ng/mL, SM-C failed to inhibit binding of [^{125}I]insulin in an RIA for insulin. SM-C was compared with IGF-I, IGF-II, SM-A, and MSA III-2 in a radioimmunoassay for somatomedin-C (SM-C RIA), a placental cell membrane radioreceptor assay for somatomedin-C (SM-C RRA), and a placental cell membrane radioreceptor assay for insulin (insulin RRA). In all these assays, SM-C and IGF-I produced identical curves of displacement. The concentrations producing 50% inhibition of radioligand binding were 0.2 ng/mL for SM-C RIA, 10 ng/mL for SM-C RRA, and 1400 ng/mL for the insulin RRA.

Somatomedin-A, IGF-II, and MSA III-2 were all less than 5% as active as SM-C or IGF-I in the SM-C RIA and less than 45% as active as SM-C/IGF-I in the SM-C RRA. In the insulin radioreceptor assay, the order of potencies was reversed. SM-C and IGF-I were only 14% as potent as IGF-II and only 43% as potent as MSA III-2. SM-A was not measured in this assay.

Dr. Raymond Hintz compared the behavior of our preparation of SM-C with IGF-I in a radioimmunoassay for the synthetic 12-residue C-peptide of IGF-I.[3] The antiserum bound [^{125}I]IGF-I and [^{125}I]SM-C equally well. Furthermore, IGF-I and SM-C produced identical curves of competition in an RIA using [^{125}I]IGF-I as the radioligand for the C-peptide antiserum. IGF-II did not cross-react with this antibody.

Biological Activity. As shown in Table III, pure SM-C stimulated confluent Balb/c 3T3 cells, pretreated with platelet-derived growth factor, to progress through the cell cycle to DNA synthesis. In the presence of 5% platelet poor plasma from a severely growth hormone deficient donor, progression activity could be demonstrated at a concentration of 1 ng/mL, and at 7 ng/mL the effect was comparable to the effect of 5% platelet poor plasma from a normal individual.

Pure SM-C also was active in vivo, since it restored active mitosis in lens epithelium of hypophysectomized frogs (*Rana pipiens* and *Rana catesbeiana*) after all proliferation had been abolished by hypophysectomy (Rothstein et al., 1980).

Discussion

Cohn fraction IV, a side product obtained in the course of purifying human serum albumin from large pools of fresh frozen human plasma, is the most abundant source of SM-C yet identified. After concentration of the initial 2 M acetic acid extract on SP-Sephadex and rechromatography on the same resin, the recovery of immunoreactive SM-C is ~50% of the theoretical quantity present in a corresponding amount of native plasma. These procedures were substituted for the previously used acid–ethanol extraction procedure because of the low yields obtained by the latter method. Simple extraction into acid, however, introduces new problems due to the coextraction of much larger quantities of inert protein. This results in considerable trailing on initial ion-exchange columns and atypical behavior on gel filtration. Chromatography of these crude extracts on Sephadex G-50 in 1 M acetic acid leads to elution of SM-C activity together with large amounts of extraneous protein in the region just preceding the salt peak (Figure 2); at later stages of purification, however, the somatomedin activity is regularly recovered at K_{av} = 0.31–0.36. On the basis of calibration curves, the latter region is characteristic of peptides with molecular weights of ~7000–9000.

Broad range isoelectric focusing served to remove a large quantity of inert acidic and neutral proteins (Figure 4) as well as the more neutral somatomedins. After removing these substances and refocusing the remainder in a narrower range, a high percentage of the residual somatomedin was recovered with an apparent p*I* of 8.2–8.5. There was, however, incomplete separation from additional somatomedin activity which focused in slightly more anodal and cathodal positions. Since only the central fractions were selected for final purification, we were unable to determine whether this spreading of zones (Figure 3B) is an artifact of the separation technique or whether it represents isohormones, deamidated molecules, or other chemically distinct somatomedins.

Prior to the introduction of high-pressure liquid chromatography, a series of separations based on size and charge properties had failed to yield a completely pure product. Reverse-phase LC, based on differences in polarity, led to the recovery of over 80% of the somatomedin activity in a sharp peak, well separated from other substances absorbing at 280 nm. The remainder of the SM-C activity eluted in slightly less polar fractions. On rechromatography, these fractions proved to consist of authentic SM-C associated with contaminating protein.

We were unable to stain 15 μg of the final SM-C preparation with either Coomassie Blue or Amido Black after analytical isoelectric focusing on polyacrylamide gel prepared sheets. Similarly, 8.75 μg of the same preparation failed to stain with Coomassie Blue after electrophoresis on polyacrylamide gel at pH 4.3. In each of these gels, the SM-C located by its immunoreactivity was confined to a single sharp band. When comparable amounts of protein from the step just prior to LC were subjected to these procedures, stained bands were clearly visible (Figures 4 and 7). Thus, the failure of the final product to stain is consistent with our interpretation that no significant amounts of contaminating proteins remain

[3] We are indebted to Dr. Raymond L. Hintz of Stanford University for permitting us to cite these previously unpublished results.

after LC. The identification of only one amino acid in the first step of the Edman degradation, the behavior of the dansylated product on TLC, and the results of $NaDodSO_4$–polyacrylamide gel electrophoresis of the fluorescamine derivative likewise attest to an apparent purity of no less than 90%.

Somatomedin-C has many chemical and biological properties in common with IGF-I. The two peptides have similar isoelectric points and are superimposable in three different radioligand systems covering a 7000-fold range of sensitivity. In these assays, both peptides are distinctly different from IGF-II and other somatomedins.

Although insufficient SM-C was available to complete the sequence analysis, the N-terminal sequence and several of the tryptic fragments were strikingly similar to IGF-I as shown in the following comparisons:

N-terminal sequence

SM-C 1-9	G-E-L-GAE
IGF 1-9	GPETLCGAE

tryptic fragments

SM-C fragment IV	R[4]GF-F
IGF 21-25	R GFYF
SM-C fragment II	R[4]DAPSTC[5]IV- -C[5]C[5]
IGF 36-48	R RAPQTG IVDEC C
SM-C fragment III	R[4]LE- -C[5]
IGF-I 56-61	R LEMYC

Thus, of the 25 residues in SM-C which could be aligned with corresponding residues in IGF-I, 22 were identical, with the three discrepancies occurring in positions corresponding to residues 37, 40, and 42 of IGF-I. Since residues 37 and 40 are part of the C-peptide of IGF-I, it is noteworthy that somatomedin-C and IGF-I reacted equally with an antibody raised against the C-peptide of IGF-I.

The sequences of our tryptic fragments I and V reveal no similarity to any portion of IGF-I. Although the quantities present in these fragments were estimated to be nearly equivalent to those in the above three peptides, the quantity of PTH amino acids derived from all fragments produced a signal to background ratio which approached the lower limit of detection.

None of the amino acids in somatomedin-C hydrolysates (Table II) were represented by fewer residues than those in IGF-I except in the case of cysteic acid where IGF-I contains six. The four cysteic acid residues found in SM-C by amino acid analysis undoubtedly represent an underestimation, however, since six such residues were identified in the sequenced tryptic fragments and additional tritium was found in another electrophoretic band which was not sequenced. Since the COOH terminus of IGF-I consists of an eight amino acid extension beyond the COOH terminus of proinsulin, it is conceivable that somatomedin-C was cleaved from a preprohormone at a different point and produced a longer extension than that of IGF-I. Although we have no direct information on this portion of the molecule, such a possibility could explain both the surplus amino acids found on amino acid analysis and the two His residues which are not present in the 70 residues of IGF-I. Much larger quantities of somatomedin-C must be purified, however, before the extent of chemical discrepancies between these two peptides can be fully clarified.

[4] Arginine is presumed on basis of cleavage technique.
[5] Identification is based on ^3H at this step.

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