

The Purification and Partial Characterization of an Insulin-Like Protein from Human Serum

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ABSTRACT A preparative scheme has been developed for the purification of a trace protein in human serum exhibiting nonsuppressible insulin-like activity (NSILA). This scheme consisted of (a) adsorption chromatography of serum utilizing the sulfonic acid polystyrene resin, Dowex 50, at pH 6.8; (b) G-200 gel filtration at pH 8.9; and (c) acrylamide gel electrophoresis in a discontinuous preparative system. Throughout all procedures, NSILA fractionated as a single molecular species approximating 90,000 mol wt. The purified protein exhibited a single band by disk gel electrophoresis, an isoelectric pH approximating 6.2, doublet bands of 90,000 and 92,000 mol wt by analytical sodium dodecyl sulfate gel electrophoresis, and a biologic specific activity approximating 50 mU/mg. Serum somatomedin (sulfation factor) activity did not fractionate with NSILA in this scheme, and partially purified NSILA did not stimulate radiosulfate uptake into hypophysectomized rat costal cartilage. This protein appears to represent the major constituent of serum NSILA: its purification and partial characterization provides the first step towards elucidation of its metabolic role.

INTRODUCTION

It is generally recognized that serum contains one or more substances, in addition to insulin and proinsulin, that exhibit insulin-like biological activity when added in vitro or injected in vivo (1-4). This insulin-like activity has been studied in several pathophysiological states and has been referred to by at least five different terms (2, 5-7). In spite of considerable research effort, the mo-

lecular nature and biological significance of this activity remains poorly defined. The principal problem resides in the fact that purification leading to physicochemical and immunochemical identification of the substances in serum exhibiting insulin-like activity has been formidable. These substances are apparently not stored in a specific organ site (8-10) and exist in serum in trace quantities. In 1968 (4) we reported physicochemical similarities among the variously described forms of serum insulin-like activity and elected to employ the term originally used by Froesch and co-workers (11), "nonsuppressible insulin-like activity" (NSILA)¹ to collectively refer to this phenomenon; that is, insulin-like activity not suppressed by the presence of excess insulin antibody. Shortly thereafter, Jakob et al. (12) further clarified this term (NSILA) by demonstrating the existence of two molecular species in serum exhibiting NSILA, one a large molecular weight protein comprising 90-95% of total serum NSILA, the other a small molecular weight protein. The small molecular weight form has been subsequently purified to three protein bands by disk gel electrophoresis and assayed at a specific activity of 450 mU/mg (13). Purification of the large molecular weight form of NSILA has not heretofore been reported.

This paper describes the preparative purification of the large molecular weight protein exhibiting NSILA and thus opens the door to the development of more precise and specific immunochemical and cell-receptor assays by which to define its biological significance.

METHODS

Fat pad segment bioassay. The purification of serum NSILA was monitored by use of a modified rat epididymal fat pad bioassay (9). This modification entailed sectioning

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¹Abbreviations used in this paper: IRI, immunoreactive insulin; NSILA, nonsuppressible insulin-like activity; SDS, sodium dodecyl sulfate; SFA, sulfation factor activity.

of the peripieral portions of the fat pad into 8–12 segments; after mixing of 80–120 segments, 3 segments at a time were blotted on filter paper and placed into preweighed incubation flasks which were again weighed before incubation. Each bioassay consisted of an insulin standard curve, 25–150 μ U/ml, plus unknown samples all assayed in triplicate. Unknown samples were equilibrated by dialysis against the bioassay buffer and diluted appropriately before bioassay. A manifold was constructed to permit simultaneous gassing of all samples. Over a 3-yr period, employing 125–170 g Texas random-bred (Sprague-Dawley derived) rats, the index of precision (λ) of this bioassay ranged from 0.08 to 0.16. The purified porcine insulin standard (PJ5589, 21.7 U/mg) used was a gift of Dr. Mary Root, Eli Lilly and Company, Indianapolis, Ind. Guinea pig insulin antiserum was developed utilizing this insulin as antigen; this antiserum was used at a final dilution of 1:1,000 (1 μ l added to 1 ml incubation medium) which suppressed the activity of 500 μ U/ml of added insulin.

Immunoreactive insulin was determined by the double antibody method modified by Soeldner and Sloane (14).

Sulfation factor assay. The hypophysectomized rat costal cartilage assay previously modified by Daughaday et al. (15) was used to assess sulfation factor activity (somatomedin-C). Hypophysectomized random-bred male rats (80 g) were obtained from Charles River Breeding Laboratories (Wilmington, Mass.) and used in the assay 14–21 days after pituitary ablation.

Starting material. Blood bank plasma was frozen and thawed in 3.2-liter quantities; the fibrin which formed was separated from the serum by filtration through glass wool at 4°C after which 400-ml volumes of serum were equilibrated with 0.01 M sodium phosphate buffer–0.14 M NaCl, pH 6.8, by dialysis in desulfurized casing against five volumes of buffer, changed every 12 h for three dialysis periods. This dialyzed serum was gently degassed and clarified by millipore filtration (0.45 μ m) before its application to the first purification step.

Column electrophoresis of serum was accomplished utilizing Sephadex G-10 as the supporting medium in a continuous buffer of Tris-EDTA-boric acid at pH 8.9 (16). The Uniphor (LKB Instruments, Inc., Rockville, Md.) column electrophoresis apparatus was employed with a 2.5 \times 45-cm gel column cooled to 4°C. After application of 3 ml serum (equilibrated by dialysis against the electrophoresis buffer) to the top (cathode end) of the column, electrophoresis was performed at a potential gradient of 300 V, 36 mA, for 22 h. Descending elution was then accomplished with electrophoresis buffer at a flow rate of 8 ml/h and monitored at 280 nm. The resulting chromatogram was arbitrarily divided into three fractions and assayed for both suppressible and NSILA after concentration in a micro-ultrafiltration apparatus (Amicon Corp., Scientific Systems Div., Lexington, Mass.) with a UM-2 membrane and dialysis against bioassay buffer.

Preparative Dowex 50 adsorption chromatography. The cationic polystyrene sulfonic acid ion-exchange resin, Dowex 50 \times 8, 50–100 mesh, was repeatedly cycled through H⁺ and Na⁺ forms by washing with 4 N HCl and 4 N NaOH to remove chromogen. After the last Na⁺ cycle, the resin was washed extensively with distilled-deionized H₂O until the effluent pH \leq 9.0 and was then poured into 3-liter preparative columns, 7.5 \times 70 cm, fitted with flow-adapters and 2.5-mm OD stainless steel inlet and outlet tubes. Operation of the columns was simplified by use of a specially constructed semiautomated system. This apparatus consisted of five, 20-liter reservoir bottles with Mariotte tubes; each flask

drained to a primary manifold containing three-way valves directing the flow to one of two secondary manifolds controlling affluent to the column. With this apparatus, two preparative columns could be developed simultaneously within 6 h. Reservoir contents were prepared biweekly and filtered (0.45 μ m membrane) before replenishment. This operation has functioned reproducibly for 3 yr at room temperature.

Preparative adsorption chromatography of 400 ml dialyzed serum was routinely performed in a stepwise fashion as follows: (a) 2 liters of phosphosaline buffer was cycled to equilibrate the resin bed. (b) 400 ml serum was applied followed by 2 additional liters of phosphosaline buffer. Non-adsorbed serum protein eluted between 900–1,400 ml effluent volume. (c) Adsorbed protein was next eluted in a stepwise fashion by cycling 0.02 N NH₄OH. This protein eluted between 900–1,300 ml, ahead of the stepwise increase in pH and was collected under constant stirring into 40 ml 0.2 N HCl at 4°C; final pH ranged from 3.4 to 3.6 and was adjusted to 4.3 with 1 N NH₄OH taking care not to exceed pH 4.5. It was necessary to maintain pH in this range since NSILA is inactivated at pH $>$ 10 (4); furthermore, if pH of the adsorbed fraction was adjusted to 4.5 or higher isoelectric precipitation of protein occurred, carrying 28–40% of the NSILA into the precipitate.² (d) 2 liters of 0.2 N NaOH was then cycled and the resin bed was maintained in NaOH overnight or until the next chromatography. (e) Before the next run, 6 liters of H₂O was cycled, followed by 2 liters phosphosaline buffer (step a). Flow rate averaged 50 ml/min.

Two adsorbed protein fractions were usually combined, representing approximately 400 mg protein in 1 liter, and were concentrated to 100 ml by ultrafiltration under N₂ employing a PM-30 membrane (Amicon Corp.) at 4°C; this fraction was then desalted by dialysis against 10⁻³ N HCl and lyophilized. The lyophilized adsorbed protein fraction has maintained stable biologic activity stored at –20°C for up to 2 yr.

Ascending preparative Sephadex G-200 filtration (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) was performed at 4°C through a 10 \times 80-cm column equilibrated in 0.05 M Tris (hydroxymethyl)aminomethane–1 M NaCl, pH 8.9. The buffer was prepared in 20-liter volumes, degassed under negative pressure, and filtered (0.22 μ m membrane) before storage and use. Solubility of the lyophilized adsorbed protein fraction was limited over the pH range of 5–8. At acid pH the gel bed was unstable, and at low ionic strength, recovery of NSILA was less than 40%; therefore, this alkaline buffer of high ionic strength was selected. Sample preparation consisted of solubilizing 500–800 mg (equivalent to 1.6 liters of starting serum) of adsorbed protein in 30–50 ml buffer under constant stirring over 4–6 h, followed by centrifugation at 10,000 *g* for 30 min at 4°C; 15–25 mg of insoluble residue was discarded and the clarified supernate was applied to the column.

The effluent fraction containing NSILA was identified by bioassay of alternate 50-ml fractions. This active fraction was then concentrated to 50–75 ml by ultrafiltration, as described above for the Dowex-adsorbed protein fraction, desalted by dialysis against deionized water at 4°C, and lyophilized. The recovery of protein in the fraction containing NSILA ranged from 15–28% of the total applied protein (*n* = 26).

Preparative acrylamide gel electrophoresis. The Uniphor apparatus was employed at 8°C; 10–30 mg of the active

² Unpublished experiments.

G-200 fraction was dissolved in 0.8–1.0 ml of 0.001 M Tris-0.008 M glycine buffer, pH 8.3, with 7% sucrose and layered onto the upper gel, dimensions 2.5×1.5 cm, composed of 2.5% total acrylamide, 0.63% bisacrylamide relative to total, polymerized with 0.5 g/100 ml riboflavin in a final buffer of 0.059 M Tris-phosphate buffer, pH 7.4. The lower gel, 2.5×4 cm, was 7% acrylamide, 0.2% bisacrylamide, polymerized with 0.14% ammonium persulfate in a final buffer of 0.375 M Tris-HCl, pH 8.9. The lower gel was pre-electrophoresed with lower gel buffer in the upper reservoir for 3 h before polymerization of the upper gel. After polymerization of the upper gel, the upper reservoir was filled with 0.005 M Tris-0.038 M glycine, pH 8.3, the lower reservoir with 0.043 M Tris-HCl, pH 8.3. During penetration of the sample into the upper gel, a potential gradient of 75 V was maintained for 90 min, after which the potential was increased to 500 V but not exceeding 20 mA. The time required to develop the electropherogram was 20–22 h. Continuous elution was accomplished with 0.06 M Tris-HCl, pH 8.3, at 0.2 ml/min.

Identification of the fraction containing NSILA was accomplished by bioassay of 0.5 ml of each 3.5-ml elution fraction. The active fraction was then concentrated by microultrafiltration with a PM-10 membrane and frozen (-20°C).

Isoelectric focusing of the active G-200 fraction was performed with the Uniphor apparatus employing the 2.5×60 -cm column at 4°C . Focusing was performed in a sucrose gradient (46.7–0.2%) containing 3 M recrystallized urea, 1% carrier ampholytes (pH 5–8), and 12 mg of the G-200 active fraction; the cathode (bottom) solutions consisted of 2% ethanolamine, 56% sucrose, 3 M urea, and the anode (top) contained 1% H_2SO_4 .

Protein was quantitated by the method of Lowry et al. (17) for the calculation of yields at various steps in the purification and for determination of biologic specific activity of the active fractions. Tris buffer, when present in the sample, was dialyzed to less than 0.1 mM before colorimetric analysis since variable interference by Tris was noted in this colorimetric method.

Analytical gel electrophoresis was performed according to the procedure described by Ritchie et al. (18) with minor modifications; analytical sodium dodecyl sulfate (SDS) electrophoresis was accomplished according to the method characterized by Weber and Osborn (19). Gels were stained with 0.5% Coomassie Blue in 25% methanol plus 10% TCA, and destained by diffusion; SDS gels were fixed in 10% TCA overnight before staining.

RESULTS

Before the development of a scheme for purification of a trace serum protein with NSILA, a series of electrophoretic separations was performed to clarify the distinction between endogenous circulating insulin and NSILA, and to assess potential alteration in total NSILA recovery after addition of insulin to serum before fractionation. The results of five such studies are summarized in Fig. 1. Panel A depicts the column electropherogram plus the observed insulin-like activity of the three arbitrarily selected fractions (I–III) resulting from electrophoresis of 3 ml serum. Total serum insulin-like activity before electrophoresis averaged 517 ± 26 $\mu\text{U}/\text{ml}$ SE ($n = 4$). Recovery of total serum activity in the

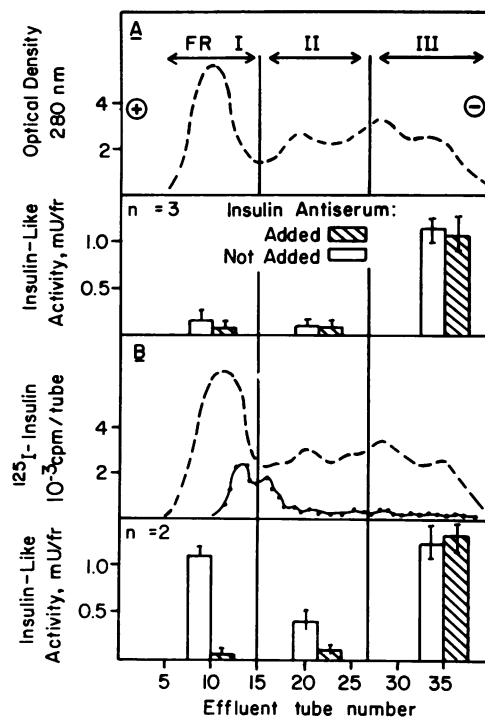


FIGURE 1 Column (G-10) electrophoresis of human serum NSILA and insulin in Tris-EDTA-boric acid buffer, pH 8.9. After electrophoretic separation and elution, the electropherograms were divided and bioassayed with and without added insulin antiserum. Panel A depicts the results of three column electrophoretic fractionations of 3 ml serum, and panel B shows the results of two identical experiments but with ^{125}I -insulin and 1.5 mU porcine insulin added before electrophoresis.

three fractions was 1.41 ± 0.05 mU ($n = 3$) or 90.9% of the activity applied to the column. 84% of this activity was not suppressed by the addition of insulin antiserum and exhibited a slow electrophoretic mobility. In Panel B, 3 ml from the same serum pool was again fractionated, but after the addition of tracer ^{125}I -insulin* plus 1.5 mU unlabeled porcine insulin. Again 95% of the total insulin-like activity was recovered ($n = 2$), 89% of the starting NSILA was recovered in fraction III, and the added insulin, assessed by both the tracer migration and bioassay, was recovered primarily in fraction I. These electrophoretic studies verified previous observations that added insulin to serum does not bind to a slower migrating serum protein (4) and demonstrated that 85–90% of the total serum insulin-like activity was comprised of NSILA exhibiting a slow electrophoretic mobility distinct from insulin.

The starting material chosen for development of a purification scheme for this NSILA was human serum

* Porcine insulin was labeled by a modification of the Hunter-Greenwood method (22).

since previous studies have demonstrated that NSILA is not stored in an identifiable organ site (8-10). Although several different fractionation schemes were plausible, the following represents the one which most consistently and economically yielded a final protein product exhibiting NSILA.

Preparative Dowex 50 adsorption chromatography. Several workers (4, 12, 20, 21) have confirmed to variable extents the original observation of Antoniadis and Gundersen (21) that a major portion of serum insulin-like activity reversibly adsorbs to Dowex 50; however, this exchange process has not been sufficiently characterized to allow design of a system on a preparative scale. Several studies were therefore performed to characterize the properties of this exchange process.

Table I lists the results of Dowex 50 chromatography of serum performed at varying pH between 6 and 8. At the lower pH range (6.19–6.37) serum NSILA was completely adsorbed, whereas, progressively less adsorption occurred at neutral and alkaline pH. At pH 7.9 only 48.6% of the serum NSILA was adsorbed. From these results it appeared that the optimal pH at which to perform Dowex 50 adsorption chromatography of serum NSILA was between 6.0 and 6.6. Since serum must be equilibrated by dialysis against the appropriate phosphosaline buffer before chromatography, a pH of 6.8 was eventually selected because of occasional troublesome precipitation of serum protein during dialysis at pH less than 6.8.

The studies characterizing optimal volume relationships during Dowex 50 serum chromatography are illustrated in Fig. 2. In this study, increasing volumes of serum from 1 to 16 ml were chromatographed through a Dowex column of 30 ml bed volume at pH 6.8, and the

adsorbed and nonadsorbed fractions were analyzed for protein content and NSILA. In the panel A of Fig. 2 the saturation curves for total adsorbed protein differs from that found for adsorbed serum NSILA: the one-half saturation for adsorbed protein (k_s) was 2 ml whereas one-half saturation for adsorbed NSILA (k_n) was 3.6 ml, indicating a higher binding capacity of serum NSILA for the resin than the other species of adsorbed serum protein.

To further characterize this binding, a Scatchard plot of the data was constructed as depicted in panel B, Fig. 2. To calculate the data required to construct this plot, a specific activity for NSILA of 50 mU/mg protein was employed. Indeed, the binding affinity of NSILA for the resin was found to be 2.7×10^4 times greater than serum protein. This comparison assumes that most serum protein potentially binds to Dowex 50, an assumption not necessarily valid; however, it emphasizes the utility in the use of Dowex 50 for adsorption of serum NSILA, and further stresses that the optimal ratio of serum volume to resin bed volume is 1.3:10. This ratio has been exceeded in most previous studies (4, 12, 20, 21). Finally, it may be estimated that 1.8×10^{13} NSILA-binding sites exist per g dry resin.

On the basis of these experiments, a preparative chromatographic system was constructed employing resin bed volumes of 3 liters, allowing adsorption chromatography of 400 ml equilibrated serum per column per day.

The results of 41 successive preparative chromatographic procedures are summarized in Fig. 3. The mean yield of adsorbed serum protein for each 400 ml processed was 192 ± 16 mg (SE) determined gravimetrically; the mean biologic specific activity was 0.48 ± 0.09 mU NSILA/mg (Table II). The heterogeneity of this ad-

TABLE I
*The Effect of pH on Dowex 50 Adsorption of Serum NSILA**

Resin-bed pH	Adsorbed protein		NSILA in adsorbed protein fraction			NSILA in nonadsorbed protein fraction
	mg	% of total serum protein	Total mU	mU/mg	% of original activity in serum	Total mU
6.19	8.96	2.85	1.61	0.180	92.0	0
6.37	7.67	2.44	1.62	0.211	92.5	0
6.95	6.49	2.07	1.43	0.220	81.7	0.50
7.40	5.80	1.85	1.27	0.218	53.7	0.70
7.90	2.57	0.82	0.85	0.311	48.6	0.76

* The Dowex 50, 50–100 mesh, resin-bed volume of 60 ml was equilibrated as described in the Methods with 0.01 M sodium phosphate-0.14 M NaCl at varying pH; 5 ml human serum was equilibrated by dialysis against the appropriate buffer before chromatography. After elution of the nonadsorbed serum protein, adsorbed protein was eluted by a stepwise increase in pH with 0.02 N NH_4OH . This adsorbed fraction (40 ml) was collected into 4 ml 0.2 N HCl at 4°C; the pH was then adjusted to 4.3. A 6-ml portion of this fraction was dialyzed against Krebs-Ringer bicarbonate buffer in preparation for bioassay; protein was determined on 0.5-ml samples in triplicate by the method of Lowry (17). Starting serum NSILA = 0.304 mU/ml or 0.0048 mU/mg protein.

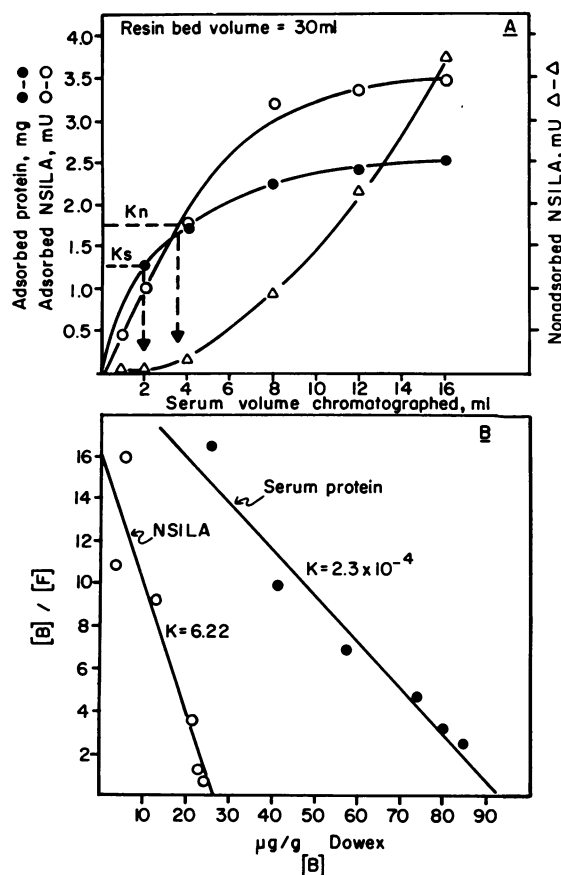


FIGURE 2 A study of the binding affinity and saturation kinetics of Dowex 50 for serum NSILA. In panel A, increasing volumes of serum were chromatographed through a resin bed volume of 30 ml (11 g Dowex dry wt) at pH 6.8. Total adsorbed protein (●) showed a half-saturation (k_s) = 2 ml; the half-saturation (○) for NSILA (k_n) = 3.6 ml. In panel B, a Scatchard plot of the data is depicted for both total adsorbed protein and adsorbed NSILA. The Y axis ([B]/[F] ratio) for adsorbed protein (not NSILA) is expressed as 10^{-3} ; the X axis for [B] NSILA as 10^3 .

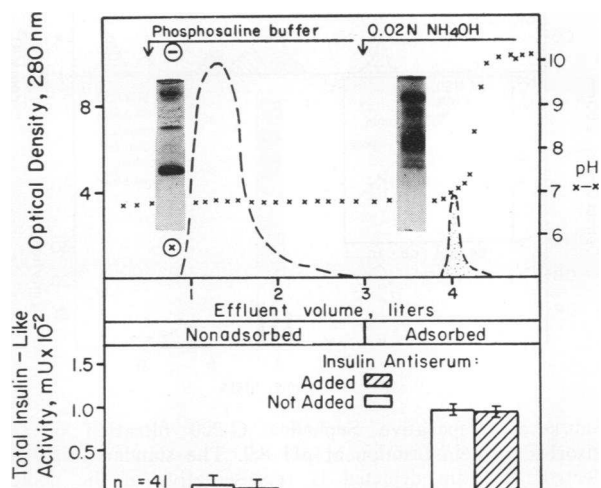


FIGURE 3 Preparative adsorption chromatography of human serum NSILA. The gel electropherograms depicted are representative of the respective nonadsorbed and adsorbed protein fractions. Bioassay of the respective fractions is illustrated below the chromatogram.

The question of the possible biological and chemical identity of somatomedin to the nonsuppressible insulin-like serum protein fractionated in this purification scheme was studied in a blind manner in two laboratories⁴ in addition to our own. A serum pool was assayed for NSILA, sulfation factor activity (SFA), and immunoreactive insulin (IRI) before and after Dowex 50 adsorption chromatography; Fig. 4 summarizes this information. NSILA decreased 82%, SFA decreased only 10%, and IRI decreased 1.2%. The decrease in serum

⁴ Sulfation factor assays were kindly performed by Dr. L. Phillips, formerly at Washington University, St. Louis, Mo., and by Dr. T. Foley, University of Pittsburgh, Pittsburgh, Pa.

sorbed protein fraction may be appreciated, in part, by comparison of the standard disk gel electropherograms of the nonadsorbed and adsorbed fractions (Fig. 3). This electrophoretic pattern was consistently observed for adsorbed fractions from different serum pools; on analytical SDS electrophoresis under reducing conditions (not shown) as many as 25 separate bands were identified ranging from 17,500 to 200,000 daltons. Bioactivity of the nonadsorbed and adsorbed fractions with and without the presence of added insulin antiserum to the bioassay medium is graphed below the representative chromatogram depicted in Fig. 3 and reveals that 95 \pm 0.06% of the recovered NSILA resides in the adsorbed fraction.

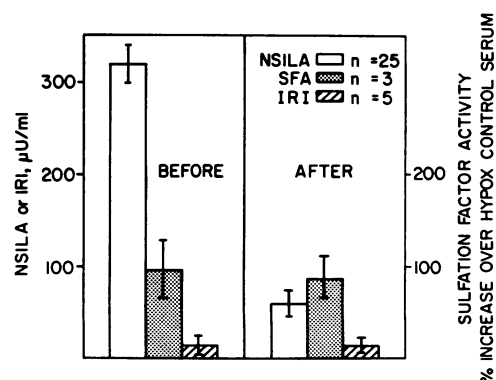


FIGURE 4 Bioactivity and insulin immunoreactivity of human serum before and after adsorption chromatography. Sulfation factor bioassay data from three laboratories are depicted.

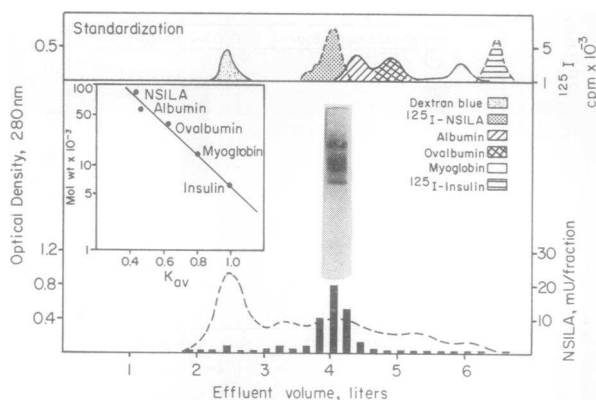


FIGURE 5 Preparative Sephadex G-200 filtration of the adsorbed protein fraction at pH 8.9. The standard disk gel electropherogram depicted is representative of the pooled G-200 fraction containing NSILA. This preparative column was standardized with known molecular weight markers as shown.

NSILA after adsorption chromatography was highly significant ($P < 0.001$); however, the decrease in SFA and IRI was not significant. This data, then, demonstrates a clear difference in the chemical behavior of NSILA and SFA, and suggests that the major portion of serum NSILA is not representative of SFA.

Preparative Sephadex G-200 filtration of the adsorbed protein fraction in an alkaline (pH 8.9) buffer of high ionic strength provided the next purification step. The standardization of this system plus a representative chromatogram is illustrated in Fig. 5. NSILA exhibited a $K_{av} = 0.447 \pm 0.005$ ($n = 4$) in this preparative system, corresponding to a mol wt approximating 90,000.⁵ To corroborate these results a radiolabeled preparation of purified NSILA⁶ was added to the standardization sample and was found to fractionate as a single peak with $K_{av} = 0.442$; this is illustrated in the upper portion of Fig. 5. The effluent fraction from 3.7 to 4.5 liters (K_{av} 0.330 – 0.526) was thereafter pooled during preparative

⁵ In previous studies with gel filtration of various NSILA preparations at neutral pH, we reported an estimated mol wt of 45,000 (4). This disagreement with the results reported herein is explained on the basis of the behavior of the hemidiaphragm bioassay which was previously employed (4); that is, protein concentrations above 2 g/100 ml are generally inhibitory to glucose uptake. In this earlier report the void volume fraction of the G-100 column contained the greatest protein content and when bioassayed was apparently inhibitory at the concentration employed. This effect resulted in an apparent shift in peak NSILA to a higher K_{av} corresponding to this lower molecular weight estimate. We failed to recognize this important point when designing the hemidiaphragm bioassay of the gel filtration chromatograms.

⁶ Purified NSILA was labeled by a modified method employing chloramine-T (22).

procedures, concentrated by ultrafiltration to 100-200 ml, desalted by dialysis, and lyophilized. The mean yield (dry wt) from 1 g applied protein (equivalent to 2.12 liters of starting serum) was 152 ± 0.04 mg which exhibited a mean biologic specific activity of 3.7 mU/mg protein. This active G-200 fraction contained five protein bands when assessed by standard disk gel electrophoresis (see inset, Fig. 5) and seven bands on analytical SDS gel electrophoresis (not shown). No SFA was found in this fraction.

Isoelectric focusing of this G-200 fraction is shown in Fig. 6. The major protein content focused at the acid end of the pH gradient, whereas, NSILA was recovered in the fraction corresponding to a pH range of 5.9-6.5. Identical blank experiments (no G-200 fraction added) did not yield NSILA. The initial peak (280 nm) is a base-line shift observed at the beginning of the elution of the dense portion of the sucrose gradient. Although these experiments served to further characterize the physico-chemical nature of NSILA, it became apparent that preparative focusing would not provide a suitable purification step because of limitations of sample size. Protein precipitation invariably occurred when larger sample sizes were focused and greater concentrations of urea or nonionic detergents partially or completely inactivated NSILA. Recovery of NSILA was estimated at only 5-10%.

Preparative acrylamide gel electrophoresis was selected for the final purification step after semipreparative pilot studies in which the G-200 fraction was electrophoresed in 5, 7, 8.5, and 10% acrylamide gels measuring 1.5×8 cm. After electrophoresis, these gels were

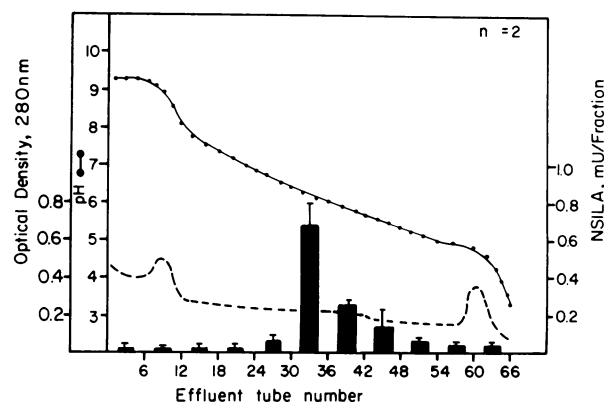


FIGURE 6 Isoelectric focusing of the active G-200 fraction (12 mg) in a pH gradient of 5-8 with 3 M urea. Initial applied potential was 400 V with 2.6 mA current flow; total running time was 42 h. The focused gradient was eluted by descending positive pressure at 1 ml/min into 3-ml fractions. After equilibration of alternate fractions by dialysis against bioassay buffer, NSILA was determined in triplicate. The mean \pm SE of replicate experiments is shown.

TABLE II
Summary of the Preparative Purification Scheme for Human Serum NSILA*

Procedure	Yield from 1 liter serum	Recovery of NSILA	Biologic sp act	Purification
	g	%	mU/mg	
Serum	—	—	0.0046±0.0008 (68)	0
Adsorption chromatography	0.472±0.006 (12)	86±9 (24)	0.48±0.09 (45)	60
G-200 filtration	0.072±0.008 (6)	84±11 (10)	3.70±0.31 (16)	804
Electrophoresis	0.001±0.0004 (3)	21±8 (5)	30 to 50 (4)	~8,700

* Means±SE are shown. The number in parentheses indicate the number of experimental observations.

frozen, sectioned, and eluted of protein by cross-electrophoresis within dialysis bags. The retentates were then centrifuged, dialyzed against bioassay buffer, and assayed for NSILA. In this manner, the crude relative mobility (R_f) for NSILA in these gels was estimated to range from 0.09 at 10% acrylamide to 0.34 at 5%. A plot of the relative mobility of NSILA and the four biologically inactive protein bands against the acrylamide concentration revealed nearly parallel lines indicating that separation was occurring largely on the basis of charge, and that the NSILA possessed a higher retardation coefficient relative to the contaminating proteins. Based upon this information a preparative acrylamide gel electrophoresis system was adopted as the final purification step.

Fig. 7 depicts the most satisfactory gel system developed to date. NSILA elutes late and recovery of applied activity has been low, ranging from 16 to 38%. Biologic activity of this fraction has been stable, stored frozen in elution buffer at -20°C , and has varied among four experiments from 30 to 50 mU/mg protein. Insulin immunoreactivity has not been found in four separate preparations. Analytical gel electrophoresis of the active fraction has revealed a single protein band

(see inset, Fig. 7) with an $R_f = 0.18$ in a 7.5% acrylamide system.

Analytical SDS gel electrophoresis of the purified NSILA was performed both in the presence and absence of mercaptoethanol, and revealed closely spaced doublet bands of 90,000 and 92,000 mol wt, in agreement with the G-200 filtration estimation (Fig. 5). It has not been possible to recover biologic activity from preparative modifications of this system; hence, it is unclear if only one or both of these protein bands exhibit NSILA.

Table II summarizes the efficiency of the purification scheme developed to date. These data indicated an 8,700-fold purification and revealed the feasibility of obtaining 1 mg of purified NSILA from 1 liter of starting serum. Employing the specific activity of 50 mU/mg this yield represented a 14% recovery⁷ of starting NSILA. There exists no purification schemes for similar trace serum proteins by which comparison of recoveries can be made; however, the low recovery in the final step indicates the need for further modification. Finally, it is important to note that all NSILA fractions derived from this purification scheme were subjected to insulin immunoassay with completely negative results throughout.

DISCUSSION

The principal significance of this work lies in the purification and partial identification of the serum protein contributing to the major portion of NSILA. This protein has been purified to a biologic specific activity of 50 mU/mg and appears as a single band on discontinuous gel electrophoresis; however, the final product probably contains two closely related proteins as evidenced by a closely spaced doublet band on SDS gel electrophoresis.

Purification of a small molecular weight serum protein exhibiting NSILA was reported by Oelz et al. in 1972 (13). This protein of 7,500 daltons was termed

⁷ The highest specific activity found for purified NSILA was 50 mU/mg and the mean serum NSILA content for the serum pool employed was 0.350 mU/ml; therefore the serum NSILA concentration approximates 7 $\mu\text{g/ml}$ (77 nM) or 7 mg/liter.

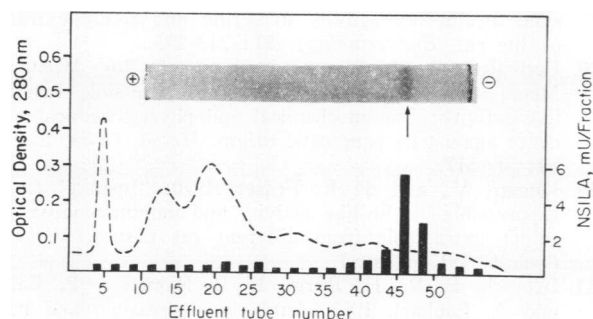


FIGURE 7 Preparative acrylamide gel electrophoresis of 20 mg of the active G-200 fraction. The first peak (280 nm) represents acrylamide polymer eluted as a result of the pre-electrophoresis step. The standard gel depicted above the active portion of the preparative electropherogram shows a single band of purified NSILA; 50 μg was applied to the analytical gel.

NSILA-S because of its solubility in acid-ethanol (12) and was estimated to contribute 5-10% of the total serum NSILA (12, 13). The remaining larger fraction, termed NSILA-P because of its precipitable behavior in acid-ethanol, was estimated to approximate 100,000 daltons (12). It is conceivable that the NSILA protein purified in this study represents this NSILA-P fraction; however, further chemical characterization of both is required before this conclusion is possible.

The biological relationship of serum NSILA to somatomedin activity has been questioned by several investigators; however, a similarity between somatomedin and the NSILA protein purified here was not apparent. Somatomedin bioassays (radiosulfate uptake) were performed in three different laboratories on all protein fractions derived from this purification scheme and the results correspond in each laboratory; i.e., somatomedin activity did not adsorb to Dowex 50 in the first purification step as does NSILA, and all G-200 fractions were negative when assayed. Furthermore, purified somatomedin (4,000-8,000 daltons) appears to possess few if any of the chemical properties found for the NSILA purified in this study (23).

A part of the identity problem in this area of insulin-like activity may reside in the effect of various purification schemes on the protein moiety being isolated. The scheme developed here differs in many respects from those developed for somatomedin and NSILA-S (23, 12). An important question raised in this respect concerns the failure to identify the small molecular weight form of NSILA (NSILA-S) in the scheme developed here. The dialysis and ultrafiltration steps were studied in detail but no NSILA was found in the filtrates. In addition, NSILA was localized in only one broad area of the column electropherogram of serum (Fig. 1). Finally, attempts to dissociate NSILA activity in the active G-200 fraction by gel filtration in 7 M urea and 5 M acetic acid-0.15 M NaCl (12, 24) failed to reveal a low molecular weight form.⁸ Further comparative speculation on the nature of serum NSILA can serve little useful purpose at this time; the solution to the questions raised will best be found once immunochemical tools become available.

In conclusion, the results of these studies verify the existence and protein nature of the major contributor of human serum NSILA and provide a scheme suitable for preparative purification. The nondissociable large molecular weight of this nonsuppressible insulin-like protein distinguishes it from smaller NSILA proteins previously isolated, NSILA-S (12) and somatomedin-C (23). Further chemical characterization plus the development of immunochemical and cell-membrane receptor methods for the more precise and specific study of this

substance should complete the necessary foundation from which to eventually elucidate its metabolic role.

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⁸ Unpublished observations.

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