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Research Article

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The Nature of Human Serum Insulin-Like Activity (ILA): Characterization of ILA in Serum and Serum Fractions Obtained by Acid-Ethanol Extraction and Adsorption Chromatography

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ABSTRACT Studies were undertaken in an attempt to clarify the apparent heterogeneous nature of human serum insulin-like activity. Methods of preparative zone electrophoresis on Pevikon, acid-ethanol extraction of trichloroacetic acid serum protein precipitates, adsorption chromatography on DEAE-cellulose and Dowex 50, gel filtration chromatography, and insulin antiserum immunoreactivity were used. The results establish the presence of a substance in serum with in vitro biological properties similar to insuln but with different physicochemical properties. The major portion of serum ILA measured by bioassay techniques can be attributed to the effects of this substance. Whereas the in vitro biological effects of this substance on muscle and adipose cells were similar to those of crystalline insulin, the substance is distinguished from insulin by: (*1*) the failure of insulin antiserum to inhibit its in vitro biological effect; (*2*) a slower electrophoretic mobility (in the gamma-beta globulin zone); and (*3*) a larger molecular weight, between 40,000 and 50,000 in these studies. It is similar to insulin since both are soluble in acid-ethanol. The results further indicate that previously described insulin-like activity in gamma-beta globulin preparations, the major portion of total serum insulin activity described in acid-ethanol extracts of serum, "bound" insulin, "atypical" insulin, and antibody nonsuppressible insulin-like activity bioassayed in diluted serum are all one and the same substance.

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INTRODUCTION

The term insulin-like activity (ILA) has evolved from the use of insulin bioassay methods which originally were thought to measure the level of insulin in serum (1, 2). However, discrepancies in data obtained from the bioassay of serum by the rat hemidiaphragm and epididymal fat pad methods suggested that these tissues were responsive to multiple factors influencing glucose uptake and utilization (3). This concept was substantiated by the development of the more specific insulin immunoassay. Normal fasting serum levels of insulin derived from immunoassay data have approximated 15–20 μU/ml (14), whereas fasting levels of "insulin" measured in vitro bioassay techniques have varied between 40 μU and 20 mU/ml (1, 3).

The nature and role of components of ILA, other than insulin, have been the subject of much speculation. This activity has been attributed to nonspecific effects of serum proteins in vitro (5), and furthermore, because serum ILA does not completely disappear after pancreatectomy and is

not altered by various physiological and pathological states, its biological significance has been questioned (6–8). Nevertheless, variations in serum ILA have been observed under certain conditions, and a possible physiological role has been implied (9, 10). By means of various protein separation procedures, several forms of ILA have been detected. One form has been found to migrate with beta and gamma globulins in serum fractionated by cold ethanol precipitation, ammonium sulfate plus DEAE-cellulose, and zone electrophoresis; however, no agreement has been reached regarding the identity of this activity (11–20). Because crystalline insulin is found with an alpha globulin in most of these procedures (17–21), it has been postulated that ILA in the beta and gamma globulins represents either binding of insulin to protein or, alternatively, other compounds with insulin-like effects (17, 18, 21).

Another form of ILA, referred to as total serum insulin activity (TSIA), is demonstrable in acid-ethanol extracts of serum (22–24). These extracts have been prepared by the hydrochloric acid–ethanol method for extraction of insulin (25) and the trichloroacetic acid–ethanol procedure for extraction of albumin (26). Because TSIA stimulated glycogen synthesis in muscle, it has been considered to represent insulin (22, 27), however, the levels of TSIA ranging from 0.8–7 mU/ml were inconsistent with the values of immunoassayable insulin in these extracts (28).

In addition, by means of the rat epididymal fat pad, ILA has been demonstrated in unfractionated serum both in the presence and absence of insulin antiserum (9, 29). The activity neutralized by insulin specific antiserum has been called "typical" or suppressible ILA, whereas the greater amount of activity not inhibited by antibody has been termed "atypical" or nonsuppressible ILA (NSILA). Recently, Bürgi, Froesch and coworkers have reported that the metabolic action of a partially purified preparation of human NSILA resembles that of insulin by several parameters tested both in vitro and in vivo (30, 31).

Following the observations of Beigelman and associates that serum ILA was no longer detectable after the passage of blood through a cation exchange resin (11), it was shown that ILA, retained on the resin, could be eluted with acid or alkaline solutions, which contrasted to the behavior of crystalline insulin (32). Based on these findings, Antoniades and colleagues concluded that the major portion of insulin in serum was in a complex form which they termed "bound" insulin (32, 33). Although "bound" insulin has been found to simulate the action of insulin in several biological systems, immunological and physicochemical data have failed to corroborate a structural relationship to insulin (34–37).

Although the exact nature of "bound" insulin, "atypical" insulin, TSIA, and NSILA has not been clarified, several lines of evidence support a direct relationship to one another as suggested by Kipnis and Stein (37) and Antoniades (10). "Bound" insulin and NSILA have been found to have electrophoretic mobilities in the beta globulin region (30, 38) and the approximate molecular weight of "bound" insulin, "atypical" insulin, and NSILA have been reported as 60,000–100,000, 30,000, and 80,000–160,000, respectively (38, 9, 30). In addition, physiological studies in humans (39) and dogs (40) have suggested that "bound" insulin and "atypical" insulin may have been formed in an extrapancreatic site. Moreover, the in vitro and in vivo metabolic effects of "bound" insulin and a partially purified preparation of NSILA are strikingly similar (41, 31). However, the results of other metabolic studies have been divergent. Glucose loading in humans apparently initiates a decrease in serum levels of "bound" insulin (42, 10), whereas "atypical"' insulin or NSILA remains unchanged (29, 43) or increases (44). Thus, although considerable data are available to infer that "bound" insulin, "atypical" insulin, TSIA, and NSILA are probably similar, the lack of comparative studies and variance in metabolic data have resulted in caution of such an interpretation (31).

Because of the lack of a clear relationship among the various forms of ILA, the following experiments were performed to determine some of the biological and physiochemical properties of ILA in human serum. ILA was measured in the presence and absence of insulin antiserum by means of the isolated rat hemidiaphragm and fat cell bioassays. The ILA in serum, acid-ethanol extracts of serum, beta and gamma globulin components of serum obtained by zone electrophoresis, and fractions of serum obtained by adsorption chromatography have been characterized by im-

munoreactivity, electrophoresis, and gel filtration. The results indicate that ILA associated with beta and gamma globulins, most TSIA measured in serum acid-ethanol extracts, "atypical" insulin, NSILA, and "bound" insulin are one substance, biologically similar to insulin but differing in immunoreactivity, electrophoretic mobility, and molecular size.

METHODS

Blood was removed from three fasting, healthy, nonobese donors at one time, the serum separated, pooled, and stored at −20°C. Throughout a 6–8 wk period, five such serum pools were obtained and stored separately.

Insulin preparations and antisera. Crystalline bovine insulin [1] used in these studies was six times recrystallized with a specific activity of 22.2 U/mg. Insulin-^{125}I and ^{131}I were prepared by a modification of the chloramine-T method of Hunter and Greenwood (45) and degradation products were removed by cellulose chromatography (46). Oxidized sulfo-B chain of insulin ($BSSO_3$) was obtained from crystalline insulin by the method of Dixon and Wardlaw (47) and radioiodinated by a procedure similar to that used for insulin.

Antiserum to a mixture of bovine and porcine insulin was developed in guinea pigs by the method of Moloney and Coval (48). Antisera at dilutions of 1:100 in the rat hemidiaphragm procedure and 1:500 in the fat cell assay suppressed completely the effect of 1000 μU of insulin per milliliter of incubation medium.

Bioassay procedures. The rat hemidiaphragm bioassay described by Vallance-Owen and Hurlock was modified as detailed previously (28). Glucose was measured in a Technicon AutoAnalyzer by the method of Hoffman (49) and the glucose uptake expressed as micrograms per milligram of dry weight during 90 min incubation. Total diaphragm glycogen, determined on an alkaline digest of the dried muscle (50) by a method using the anthrone reagent (51), was expressed as micrograms of glycogen/milligram of dry muscle during 90 min incubation. Statistical comparison of group means was performed where appropriate, and SEM was calculated by conventional techniques (52).

The rat epididymal fat cell bioassay was identical to that reported by Gliemann (53). Lyophilized acid-ethanol–extracted serum albumin and beta globulin prepared by zone electrophoresis were added to the incubation medium in varying amounts. The final protein concentration was adjusted to 40 mg/ml by varying the quantity of albumin.[2]

Acid-ethanol extraction of serum. Serum was extracted by a modification (28) of the trichloracetic acid (TCA)–ethanol procedure described by Debro, Tarver, and Korner (26). Although albumin is the major protein extracted by this procedure (26), other components of serum have been detected [3] (54) and for descriptive convenience, the extract will be referred to as "albumin." The extract in 1% TCA–95% ethanol was dialyzed in boiled, grade 27, Visking cellophane casing (28) for 24 hr against running tap water, 48 hr in six consecutive 10 liters of deionized water at 4°C, and lyophilized. In some experiments the ILA in these acid-ethanol extracts was partialy separated by isoelectric precipitation of albumin. This was accomplished by adjusting the pH of the TCA-ethanol extract to 4.9 with 5 N ammonium hydroxide and precipitated albumin was separated by centrifugation. The resulting supernatant was lyophilized after extensive dialysis as detailed above. The average yield of supernatant material was 350 mg/100 ml of serum extracted.

Anionic exchange chromatography. DEAE-cellulose [4] was prepared for use by alternate washing with 0.1 N sodium hydroxide, 0.1 N phosphoric acid, and equilibration of the hydroxide form in 0.001 M phosphate buffer, pH 8. Serum (20 ml) was applied to a column (52 × 2 cm) containing 30 g of cellulose and fractional elution of serum protein was performed with 0.001 M phosphate buffer, pH 8 (Fraction I), and 0.1 M phosphate buffer, pH 7 (Fraction II), at flow rates of 100 ml/hr. Fractions I and II were lyophilized after extensive dialysis in boiled, grade 18, Visking cellophane casing against running tap water and deionized water.

Serum (20 ml) containing 165,000 cpm of insulin-^{131}I (SA was 250 mc/mg) was subjected to DEAE-cellulose chromatography under similar conditions as above, except after elution of Fraction II, the cellulose was washed with 6 M acetic acid (Fraction III). Radioactivity in aliquots from the seperate fractions was measured in an autogamma well counter.

Bovine crystalline insulin (25 mg) dissolved in 1.0 ml of 0.1 N HCl, was added to 10 ml of 0.001 M phosphate buffer, pH 8.0, and the final pH was adjusted to 8 with NaOH. The solution was applied to the DEAE-cellulose column and eluted with buffers and acetic acid as described. Protein in the collected fractions was determined by absorbency at 280 mμ and the fractions were desalted by dialysis and lyophilized.

Cationic exchange chromatography. Dowex 50 × 8,[5] 100–200 mesh, was prepared by the procedure outlined by Antoniades and Gundersen (55) and was used in the Na^+ cycle, pH 6.6 ± 0.2. Chromatography was performed in glass columns (25 × 3.5 cm) with an amount of wet resin equivalent to serum (v/v). Serum was passed through the resin at flow rates between 3–5 ml/min at room temperature, and the resin was subsequently washed with 3 volumes of cold 0.15 M NaCl. The combined serum effluent and wash were desalted by dialysis and lyophilized. Trichloracetic acid–ethanol extraction of this fraction was performed by dissolving 700 mg of the ly-

[1] Donated by Boots Pure Drug Company, Ltd., Nottingham, England.

[2] Fraction V, Lot B 23709 or B 23809, Armour Pharmaceutical Co., Kankakee, Ill.

[3] Poffenbarger, P. L., and J. W. Ensinck. Unpublished observations.

[4] Cal Biochem, Los Angeles, Calif.

[5] J. T. Baker Chemical Company, Phillipsburg, N. J.

ophilized protein in 10 ml of Gey and Gey buffer at pH 8 (56). Extraction was then performed as described for serum (28).

After passage of serum, the protein retained on the resin was eluted with 2 volumes of 0.02 N NH_4OH into an equivalent quantity of 0.2 N sulfuric acid under constant stirring. Flow rates were maintained at 15–20 ml/min and the pH of the collected solution was adjusted to 7.4, and the solution was subsequently lyophilized. Protein concentration, determined by a modification of the method of Lowry, Rosebrough, Farr, and Randall (57) varied between 200–300 μg/ml, calculated on the basis of equivalence to volume of serum applied to the resin. 24 hr before assay by the isolated rat hemidiaphragm method, a weighed amount of the lyophilized alkaline eluate, equivalent to the calculated concentration in 10 ml of serum, was dissolved in 5–6 ml of Gey and Gey buffer and dialyzed in boiled, grade 18, Visking casing against 100 volumes of Gey and Gey buffer containing glucose (3 mg/ml). The retentate was adjusted to a final volume of 10 ml with buffer plus glucose. Preparation of the alkaline eluate for assay in the isolated rat epididymal fat cell system was performed in similar fashion except that Krebs-Ringer bicarbonate buffer with glucose (0.1 mg/ml) was used. Albumin (50 mg/ml) and glucose-1-^{14}C (0.05 μc/incubation flask) were added after completion of dialysis.

Preparative zone electrophoresis. Electrophoresis was performed in a block (50.5 × 37.5 × 1.5 cm) of Pevikon,[6] equilibrated in 0.35 M Tris-EDTA-borate buffer, pH 9 (58). Serum was equilibrated by dialysis in buffer and applied to the origin (1 × 35 cm) located 12 cm from the cathode. In the experiments in which acid-ethanol–extracted "albumin," supernatant material, and alkaline eluate protein were subjected to electrophoresis, serum was run in parallel as a control for division of the block. Electrophoresis was performed at 4°C for 20–22 hr with a potential gradient of 8 v/cm and 100–120 ma current flow. Subsequently, the block was cut into 1 cm sections and protein eluted with 30 ml of 0.01 M Tris-EDTA-borate buffer. The protein content was determined by absorbence at 280 mμ. The four major protein fractions (gamma, beta, $alpha_2$ globulin, and $alpha_1$ globulin-albumin) were segregated, desalted by dialysis, and lyophilized. Average recovery varied from 80 to 92% of the original protein fractions of serum, determined by quantitative electrophoresis on cellulose acetate.

Gel filtration. Ascending gel filtration was performed at room temperature through a column (180 × 2 cm) of Sephadex G-100[7] in 0.15 M phosphate buffer, pH 7.4, at flow rates varying from 25 to 30 ml/hr. The column was calibrated using the following compounds of known molecular weight: Dextran 2000[7] (2 million, average mol wt); human crystalline albumin[8] (69,000 mol wt); bovine ovalbumin[7] (44,000 mol wt); bovine myoglobin[9] (16,900 mol wt); bovine crystalline insulin[10] (12,000 mol wt); and radioiodinated sulfo-B chain of insulin (4000 mol wt, specific activity 200 mc/mg). 20 mg of each protein and 2×10^6 cpm of $BSSO_8$-^{131}I were dissolved in 5 ml of 0.15 M phosphate buffer, pH 7.4, and separately filtered. Protein content of each fraction (6 ml/tube) was determined by the absorbence at 280 mμ and radioactivity was measured in an autogamma well counter. Distribution coefficients (K_d) were determined for each protein according to the method outlined by Grannath and Flodin (59).

Protein samples from zone electrophoresis and acid-ethanol extraction of serum were applied separately to the column in 5 ml of phosphate buffer. Alkaline eluate salt (120 mg/5 ml of water) was equilibrated in buffer before filtration. Serum (20 ml) was equilibrated in buffer by dialysis and lyophilized. The powder was dissolved in 5 ml of water and applied to the column. After determination of absorbence at 280 mμ in the eluted fractions, the peaks were segregated into five fractions, desalted by dialysis, and lyophilized.

RESULTS

ILA in serum fractionated by zone electrophoresis. Resolution of serum protein into four fractions was obtained by electrophoresis of 20 ml of serum from each of four separate serum pools on Pevikon in 0.35 M Tris-EDTA-borate buffer at pH 9 (Fig. 1). Although a distinct $alpha_1$ globulin was not observed, albumin preparations from the Pevikon block revealed the $alpha_1$ migrating component on cellulose acetate electrophoresis (not shown). Insulin-^{125}I added to serum migrated in the $alpha_1$ globulin–albumin zone. As shown in Figure 1, significant stimulation of glucose uptake in the rat hemidiaphragm was only observed with fractions from the beta and gamma globulin regions with percentage increases in uptake of 80 and 48%, respectively.

The effect of guinea pig anti-insulin serum on the stimulation of glucose uptake produced by the gamma and beta globulins from three separate serum pools is shown in Fig. 2. Excess antiserum (dilution 1 : 100) was added before incubation and neutralized the effect of 1000 μU of insulin per milliliter. However, the gamma-beta globulin preparations at protein concentrations of 10 mg/ml contained ILA, which on bioassay was equivalent to 100–500 μU of insulin, yet the ILA was not suppressed by the addition of antiserum. The inability to detect any ILA in the $alpha_1$ globulin–albumin region where crystalline insulin migrates

[6] Superfostat Bolaget, Stockholm, Sweden.
[7] Pharmacia Fine Chemicals, Inc., Piscataway, N. J.
[8] Pentex Company, Philadelphia, Penna.
[9] Mann Research Laboratories, New York.
[10] Sigma Chemical Company, St. Louis, Mo.

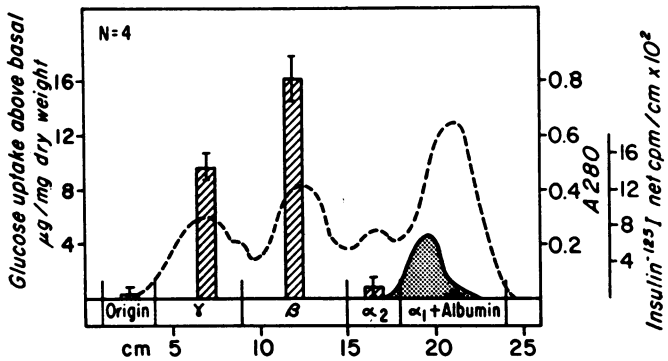

FIGURE 1 Electrophoretic mobility of serum ILA and insulin-^{125}I. Serum (20 ml) was submitted to electrophoresis on Pevikon in 0.35 M Tris-EDTA-borate buffer, pH 9. The resulting protein fractions were combined into four pools (+ the origin) as shown by the short vertical lines. Each pool was assayed by the rat hemidiaphragm response. Mean net glucose uptake of four experiments (four hemidiaphragms per experimental group) is represented by the bars. Vertical lines indicate SEM. Gamma, beta, and alpha$_2$ globulins were assayed at 12.5 mg/ml, alpha$_1$ globulin–albumin at 40 mg/ml. The electrophoretic migration of insulin-^{125}I, added to 20 ml of serum (1000 cpm/ml) in one experiment, is shown as the dotted area.

(21) may be explained by the levels of insulin in fasting serum, measured immunologically (10–20 µU/ml) (1, 4), which are below the sensitivity of the hemidiaphragm assay. Thus, these studies demonstrated that a substance with an electrophoretic mobility corresponding to beta and gamma globulins was capable, like insulin, of stimulating glucose uptake in muscle. However, this substance(s) differs from insulin in its electrophoretic mobility and its inability to be neutralized by insulin specific antiserum.

ILA in acid-ethanol extracts of serum. The stimulation of glucose uptake into rat hemidiaphragm produced by TCA–ethanol extracts of serum (four separate pools) is illustrated in Fig. 3. It is evident that ILA was extracted into 1% TCA–95% ethanol with serum albumin and that this ILA was not inhibited by the addition of insulin specific antiserum. In order to characterize further the ILA in the "albumin" preparations, the procedure of isoelectric precipitation was applied. After isoelectric precipitation of albumin in TCA-ethanol serum extracts, the supernatant was found to contain most of the ILA and no suppression of activity was observed in the presence of insulin antibody (Fig. 3). Although complete sepa-

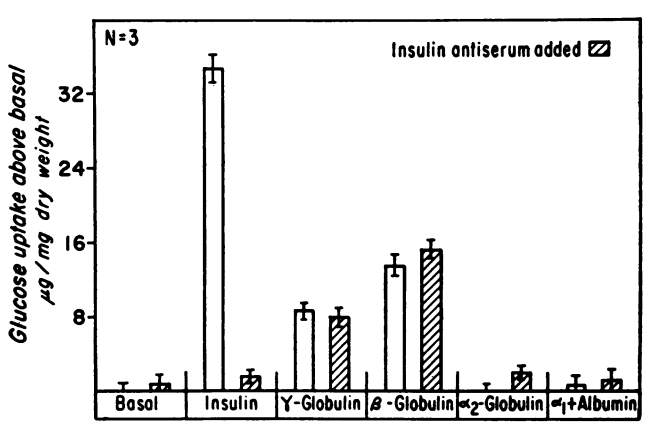

FIGURE 2 Effect of insulin antiserum on ILA of serum proteins prepared by zone electrophoresis. Protein fractions were assayed by the rat hemidiaphragm response in the presence and absence of insulin antiserum (1:100 dilution). Concentration of gamma, beta, and alpha$_2$ globulin was 10 mg/ml; alpha$_1$ globulin–albumin was 40 mg/ml. Insulin concentration was 1000 µU/ml. Bars represent the mean net glucose uptake of three experiments (four hemidiaphragms per experimental group) and vertical lines indicate SEM.

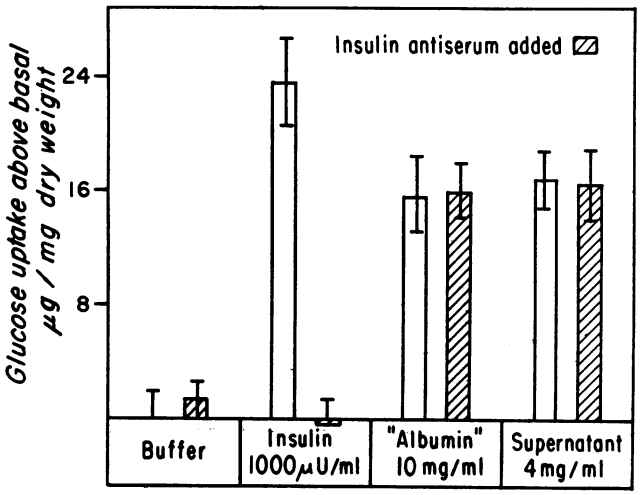

FIGURE 3 Stimulation of glucose uptake into the rat hemidiaphragm by "albumin" and supernatant material. For preparation of "albumin" and supernatant material, refer to Methods. Insulin antiserum dilution was 1 : 100. Bars represent mean net glucose uptake in four experiments (three hemidiaphragms per experimental group) and vertical lines indicate SEM.

ration of ILA from albumin was not achieved by this procedure, a two- to three-fold purification was accomplished. Thus, as in the beta and gamma globulin fractions from zone electrophoresis of serum, ILA that was not suppressed by insulin antiserum was also extracted into acid-ethanol.

DEAE-cellulose chromatography of unmodified serum ILA and acid-ethanol–extracted serum ILA. In Table I are shown the effects, on glucose uptake in isolated rat muscle, of serum (Experiment 1), "albumin" (Experiment 2), and supernatant material (Experiment 3) fractionated on DEAE-cellulose. Fractions I and II for each of the separate experiments were tested with and without insulin specific antiserum.

In Experiment I, in which serum was chromato-

TABLE I

DEAE-Cellulose Chromatography of ILA in Serum and Acid–Ethanol Extracts of Serum

Experiment	Experimental group	Chromatographic fraction*	Protein concentration	Glucose Uptake‡	
				Fraction alone	Antiserum added§
			mg/ml	*μg/mg dry wt*	
1	Buffer			20.98 ± 1.46	
	Insulin‖			48.64 ± 1.70	21.72 ± 2.42
	Serum	I	10	23.78 ± 2.84	23.90 ± 1.18¶
		II	40	29.58 ± 1.18	29.46 ± 1.12**
2	Buffer			20.12 ± 0.58	
	Insulin			47.66 ± 1.46	22.32 ± 1.78
	"Albumin"‡‡	I	0.25	18.58 ± 1.16	19.12 ± 1.56¶
		II	40	26.34 ± 2.08	26.08 ± 1.16**
3	Buffer			16.50 ± 0.64	
	Insulin			44.10 ± 2.30	18.18 ± 2.12
	Supernatant material‡‡	I	0.10	16.76 ± 1.82	18.14 ± 1.38¶
		II	2	38.94 ± 1.08	38.68 ± 0.90§§

* Fraction I was eluted with 0.001 M phosphate buffer, pH 8; Fraction II with 0.1 M phosphate buffer, pH 7.

‡ Each value represents the mean uptake of three hemidiaphragms ± SD.

§ Insulin antiserum dilution is 1:100.

‖ Insulin concentration was 1000 μU/ml in the three experiments.

¶ Values are not significantly different (> 0.05) from uptake in buffer alone.

** Difference between uptake of fraction + antiserum and that in buffer alone is significant at $P < 0.02$.

‡‡ Refer to Methods for preparation of "albumin" and supernatant materials.

§§ Difference between uptake of fraction + antiserum and that in buffer alone is significant at $P < 0.001$.

graphed, Fraction I consisted of gamma globulins and Fraction II contained albumin, alpha and beta globulins as determined by cellulose acetate electrophoresis. In Experiments 2 and 3, in which 1.5 g "albumin" and 80 mg of supernatant material from acid-ethanol extracts of serum were subjected to chromatography, only 5 and 2 mg of protein were recovered, respectively, in Fraction I, whereas Fraction II contained the major amount of protein.

As is apparent from Table I, a significant increase in glucose uptake above that in buffer alone was observed only with the protein in Fraction II from each of the three experimental groups ($P <$ 0.02, $<$ 0.02, and $<$ 0.001, respectively). In addition, the ILA in these fractions was not neutralized by insulin specific antiserum. The low amounts of ILA detected in Fraction II in Experiments 1 and 2, in which the protein concentrations were 40 mg/ml, may be due to the presence of an inhibitor associated with albumin as described previously (28). Thus, nonsuppressible insulin-like activity in serum, in acid-ethanol extracts of serum ("albumin"), and in its concentrate in the supernatant fraction was eluted from DEAE cellulose under identical conditions. In contrast to the behavior of serum ILA, 95% of insulin-^{131}I in serum and virtually all crystalline insulin was retained on DEAE-cellulose and approximately 50% of both was eluted with 6 N acetic acid (Table II). The experimental yields of ILA and insulin from DEAE-cellulose chromatography were below the expected values and probably reflect losses from adsorption to cellulose and from processing of the effluent fractions for assay.

TABLE II

*DEAE-Cellulose Chromatography of Crystalline Insulin and Insulin-^{131}I**

Chromatographic fraction	Insulin-^{131}I	Insulin
	cpm	*mg*
I	500	0
II	7,700	0
III	71,300	14
Per cent recovery	48	56

* Insulin-^{131}I (165,000 cpm) in 20 ml of serum and 25 mg of crystalline insulin were chromatographed on DEAE-cellulose. Fraction I was eluted with 0.001 M phosphate buffer, pH 8; Fraction II with 0.1 M phosphate buffer, pH 7; and Fraction III with 6 N acetic acid.

Dowex 50 chromatography of serum ILA. In Table III are shown the results of studies designed to determine the chromatographic behavior of ILA on Dowex 50 × 8 (100–200 mesh). Equal volumes of pooled human serum were passed through varying volumes of Dowex 50 equilibrated in 0.15 M NaCl. 99% of the total protein was recovered in the NaCl wash. This protein fraction was assayed directly at 10 mg and 70 mg/ml. In addition, this fraction was extracted with TCA-ethanol and the "albumin" assayed at 10 mg/ml. The material retained on the resin was eluted with alkali (alkaline eluate) and assayed at concentrations approximating those in the original serum. In contrast to the studies of Antoniades and Gunder-

TABLE III

*Dowex 50 Chromatography of Serum ILA**

Resin volume	Serum volume	Glucose uptake above basal‡							
		NaCl wash§				Acid-ethanol extract of NaCl wash		Alkaline eluate‖	
ml	*ml*	*10 mg/ml*	*P*	*70 mg/ml*	*P*	*10 mg/ml*	*P*	*0.25 mg/ml*	*P*
10	20	7.40 ± 0.58	< 0.05	8.52 ± 1.28	< 0.02	5.66 ± 1.14	< 0.05	13.56 ± 3.00	< 0.001
20	20	2.08 ± 0.36	< 0.05	5.32 ± 0.58	< 0.05	1.94 ± 1.28	NS	22.58 ± 3.34	< 0.001
30	20	1.20 ± 1.20	NS	4.78 ± 1.68	< 0.05	−0.42 ± 0.54	NS	23.90 ± 2.58	< 0.001
40	20	2.48 ± 0.76	NS	4.52 ± 0.92	< 0.05	1.56 ± 1.46	NS	25.32 ± 2.04	< 0.001

* Chromatography of serum was performed through varying volumes of resin.

‡ Glucose uptake is expressed as μg/mg of dry muscle and represents the mean net uptake of three hemidiaphragms ± SD. P values indicate levels of significance of the uptake in buffer alone (basal). NS refers to $p > .05$.

§ NaCl wash refers to serum proteins passed through the resin combined with the wash in 0.15 M NaCl.

‖ Alkaline eluate refers to the fraction eluted from the resin with 0.02 N NH_4OH.

TABLE IV

*Reproducibility of Dowex 50 Chromatographic Separation of ILA in Serum**

Insulin		Alkaline eluate‡			
	Glucose uptake above basal	Determination		Glucose uptake above basal	Specific activity
μU/ml			*μg protein/ml*		*μU ILA/mg protein*
100	6.62 ± 1.12	1	250	18.10 ± 3.02	2000
200	12.06 ± 1.12	2	260	19.84 ± 2.40	2300
400	16.68 ± 2.50	3	265	18.62 ± 1.82	2000
600	19.30 ± 3.40	4	260	18.20 ± 2.24	2000
800	21.24 ± 2.52				

* Dowex 50 × −8 chromatography of a pool of serum was performed at a resin to serum volume ratio of 1:1 on four separate occasions. All fractions were assayed by the rat hemidiaphragm response simultaneously with the insulin dose response curve. Glucose uptake is expressed as μg/mg of dry muscle and represents the mean net uptake of three hemidiaphragms ± SD.

‡ Alkaline eluate refers to the fraction eluted from the resin with 0.02 N NH_4OH. The protein concentration of this fraction is equivalent to the amount of protein eluted per milliliter of serum applied.

sen (60), the bioassay of the alkaline eluate by the rat hemidiaphragm procedure did not include the presence of adipose tissue extracts. As indicated in Table III, ILA was detected in the alkaline eluate from the Dowex column and maximum retention of ILA on the resin was observed at resin : serum volume ratios of 1 : 1 or greater. At a resin : serum volume ratio of 1 : 2, 43% less ILA was found in the alkaline eluate and correspondingly more ILA was recovered in the serum proteins which were not retained on the resin (NaCl wash). It is also apparent that, after acid-ethanol extraction of the serum proteins in the NaCl wash, additional ILA was not demonstrated. These data, therefore, are compatible with the conclusion that most of the serum ILA is retained on the cationic exchange resin and that ILA is not due to a nonspecific effect of acid-ethanol on serum proteins.

In Table IV is shown the ILA in the alkaline eluate from four replicate experiments using pooled serum at resin : serum volume ratios of 1 : 1. It is evident that, within the limits of precision of the rat hemidiaphragm procedure, the recovery of ILA in the alkaline eluate from Dowex 50 × 8 is reasonably reproducible. In contrast, considerable variation in ILA was encountered in alkaline

TABLE V

*Variation of Alkaline Eluate ILA in Sera from Different Human Subjects**

Insulin		Alkaline eluate‡			
	Glucose uptake above basal	Lot		Glucose uptake above basal	Specific activity
μU/ml			*μg protein/ml*		*μU ILA/mg protein*
200	6.98 ± 2.66	1	163	16.10 ± 1.70	3060
400	14.30 ± 0.80	2	275	12.46 ± 1.72	1320
600	17.82 ± 1.52	3	294	6.30 ± 3.52	610
800	22.54 ± 2.80	4	250	13.46 ± 1.90	1560
1000	24.54 ± 1.40	5	430	18.44 ± 1.64	1418

* Dowex 50 × −8 chromatography of serum from five separate lots was performed at a resin to serum volume ratio of 1:1. All fractions were assayed by the rat hemidiaphragm response simultaneously with the insulin dose response curve. Glucose uptake is expressed as in Table IV.

‡ Alkaline eluate refers to the fraction eluted from the resin with 0.02 N NH_4OH. The protein concentration of this fraction is equivalent to the amount of protein eluted per milliliter of serum applied.

TABLE VI

Effect of Insulin Antiserum on Biological Activity of Serum ILA Separated by Dowex 50 Chromatography

Fraction*	Protein concentration	Glucose uptake‡			
		Fraction alone	*P*	Fraction plus antiserum	*P*
	mg/ml	*μg/mg dry wt*			
Buffer (basal)		22.18 ± 2.20			
Insulin		49.20 ± 3.26	< 0.001	24.18 ± 1.84	NS
NaCl wash	70	28.44 ± 1.98	< 0.05	24.56 ± 3.62	NS
Alkaline eluate	0.20	37.06 ± 3.58	< 0.001	39.14 ± 0.82	< 0.001

* For description of NaCl wash and alkaline eluate fractions, refer to Table III. Insulin concentration is 1000 μU/ml and insulin antiserum dilution is 1:100.

‡ Each value represents the mean glucose uptake of three hemidiaphragms ± SD. The *P* values refer to the level of significance of difference between glucose uptake in buffer alone and that in the corresponding fractions.

eluates obtained from five individual lots of serum (Table V). The protein concentration varied between 163 and 430 μg/ml and the specific activities between 610 and 3060 μU equivalents of insulin per milligram of eluate protein. The average of five determinations was 1600 μU/mg of protein which is a two- to three-fold greater specific activity than that reported previously in assay systems employing adipose tissue extracts (34, 41, 60).

Table VI shows the effect on glucose uptake in muscle of serum proteins in the NaCl wash and in the alkaline eluate in the presence of insulin antiserum. A small but significant ($P < 0.05$) increase in glucose uptake was observed with the proteins of the NaCl wash. After the addition of antiserum, the glucose uptake was not significantly different from the uptake under basal conditions or in the NaCl fraction without antiserum. Therefore, although it is difficult to be certain that the ILA was suppressed, it does indicate that little if any nonsuppressible ILA was present in the NaCl wash. In contrast, the large amount of ILA in the alkaline eluate was not neutralized by insulin specific antiserum, and by means of radioimmunoassay, no immunologically measurable insulin could be detected.

Dowex 50 chromatography of the ILA in "albumin" preparations. "Albumin" prepared by TCA-ethanol extraction of serum was passed through Dowex 50 × − 8 under conditions described for serum. In Table VII is illustrated the effect on glucose uptake in muscle of the proteins in the NaCl wash, and in the alkaline eluate in the presence and absence of insulin antiserum. The ILA in "albumin" that was not neutralized by insulin specific antiserum was retained on the resin, which coincided with the behavior of ILA from

TABLE VII

Effect of Insulin Antiserum on Biological Activity of "Albumin"-ILA Separated by Dowex 50 Chromatography

Fraction*	Protein concentration	Glucose uptake‡			
		Fraction alone	*P*	Fraction plus antiserum	*P*
	mg/ml	*μg/mg dry wt*			
Buffer (basal)		22.18 ± 2.20			
Insulin		49.20 ± 3.26	< 0.001	24.18 ± 1.84	NS
NaCl wash	10	24.88 ± 2.08	NS	24.26 ± 2.44	NS
Alkaline eluate	0.32	32.58 ± 1.86	< 0.001	31.92 ± 2.64	< 0.001

* "Albumin" (1 g in 20 ml of Gey and Gey buffer, pH 8) was chromatographed on Dowex 50 × −8 (as described for serum). For description of NaCl wash and alkaline eluate fractions, refer to Table III. Insulin concentration is 1000 μU/ml.

‡ The data are presented as in Table VI.

serum subjected to Dowex chromatography (Table III). Thus, it seems reasonable to conclude the acid-ethanol extraction of serum did not alter the property of this insulin-like substance to adsorb to the Dowex 50 resin.

Dose response relationships of ILA in various protein fractions. Fig. 4 and 5 show the dose response curves for glucose uptake and glycogen synthesis in the rat hemidiaphragm and glucose oxidation in the adipose cell assay for the different protein fractions containing ILA in the presence of insulin antiserum. The effect of crystalline insulin is included for comparison. It is evident that the ILA in all of the protein fractions not only caused glucose uptake in muscle but also glycogen deposition (Fig. 4). The response to insu-

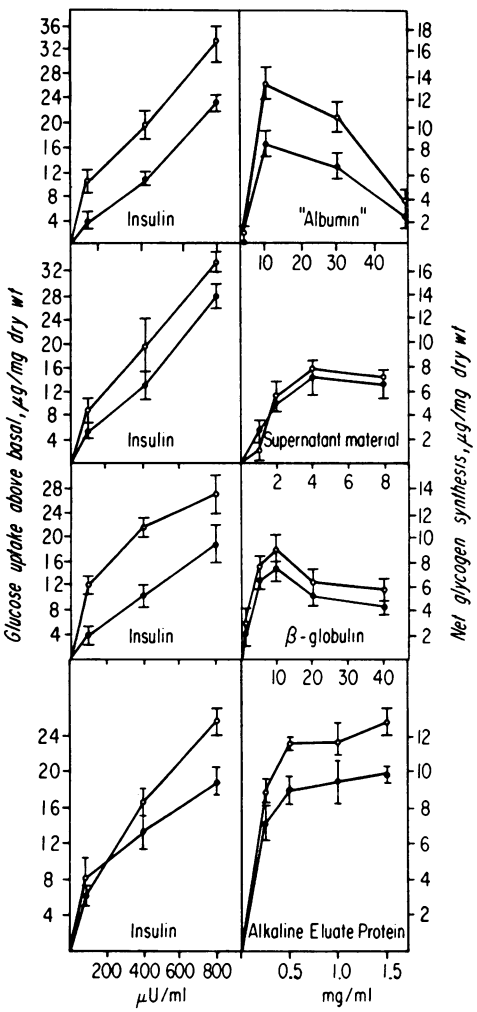

FIGURE 4 The effect of varying concentrations of acid-ethanol extracts of serum, beta globulin, and alkaline eluate on glucose uptake and glycogen synthesis in rat hemidiaphragm. For preparation of "albumin," supernatant material, beta globulin, and alkaline eluate refer to Methods. The points for glucose (○—○) and glycogen (●—●) represent means of three experiments for the acid-ethanol extracts, two experiments for the beta globulin fraction, and two experiments for the alkaline eluate protein from separate serum pools. Vertical lines indicate SEM. Insulin dose response curves, which are shown for comparison, were performed at the same time.

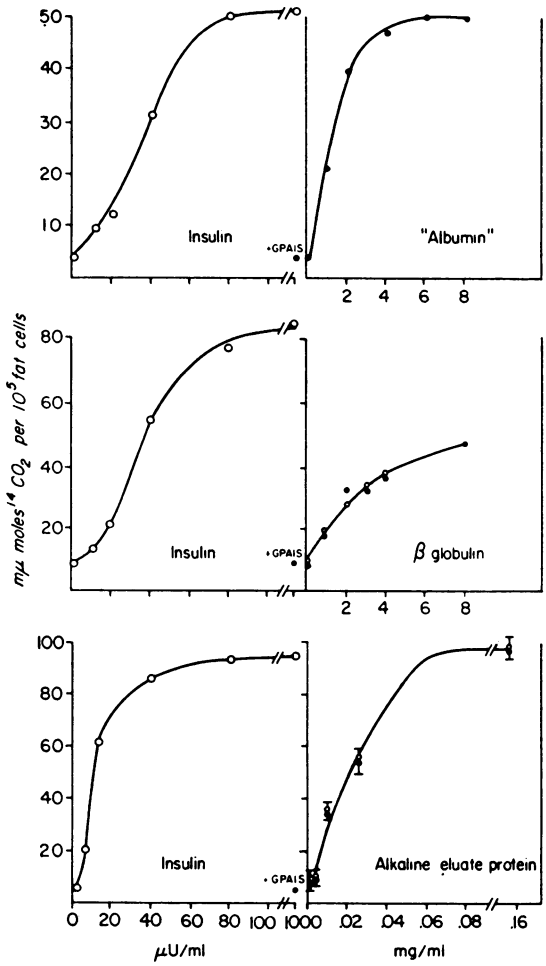

FIGURE 5 The effect of varying concentrations of acid-ethanol extracts of serum, beta globulin, and alkaline eluate on glucose 1-^{14}C oxidation in isolated fat cells. For preparation of "albumin," beta globulin, and alkaline eluate refer to Methods. The various fractions were assayed in the absence (○—○) and presence (●—●) of insulin antiserum (G-PAIS) at 1 : 500 dilution. Each point represents the mean of duplicate determinations for the "albumin" and beta globulins and triplicate determinations for the alkaline eluate protein with SD indicated by vertical lines. Each preparation is shown with the corresponding insulin dose response.

lin by both of these parameters (Fig. 4) approached a maximum at a concentration between 800 and 1000 μU/ml, whereas with the exception of the ILA in the alkaline eluate, the increase of glucose uptake and glycogen synthesis by these fractions did not achieve the maximum effect of insulin. The greatest effect (Fig. 4) occurred with beta globulin at 10 mg/ml, supernatant material at 4 mg/ml, and "albumin" at 10 mg/ml. Although beta globulin and supernatant material at these concentrations approximated the calculated concentration in serum, the maximum effect of albumin was seen at one-fourth of its serum concentration. With increasing concentration, less apparent ILA was detected, which suggests that inhibition of the ILA had occurred at higher concentrations of ILA in fractionated serum by the hemidiaphragm bioassay.

In contrast to the evidence of inhibition of ILA with the above fractions, the ILA in the alkaline eluate protein did not appear to be inhibited since it achieved the maximum effect observed with 800 μU of insulin. That this lack of inhibition is not the result of lesser amounts of protein in the alkaline eluate is indicated by the observations that purified albumin at concentrations of 50 mg/ml does not inhibit the effect of insulin in the hemidiaphragm bioassay (28).

Fig. 5 shows the dose response relationships for ILA in "albumin" beta globulin and alkaline eluate protein as assayed in a fat cell bioassay system with and without insulin antiserum. Because of the increased sensitivity of this assay, lower concentrations of the protein fractions were possible. From the curvilinear dose response relationships for the ILA in "albumin" and the alkaline eluate, which were similar in contour to those of insulin, it is concluded that no inhibition was present, whereas the ILA in the beta globulin protein tended to plateau below the insulin response suggesting the presence of an inhibitor in this fraction. Despite the differences in dose response relationships on muscle and adipose tissue, the ILA in these derivatives of serum exerted a qualitatively similar biological effect in both assay systems.

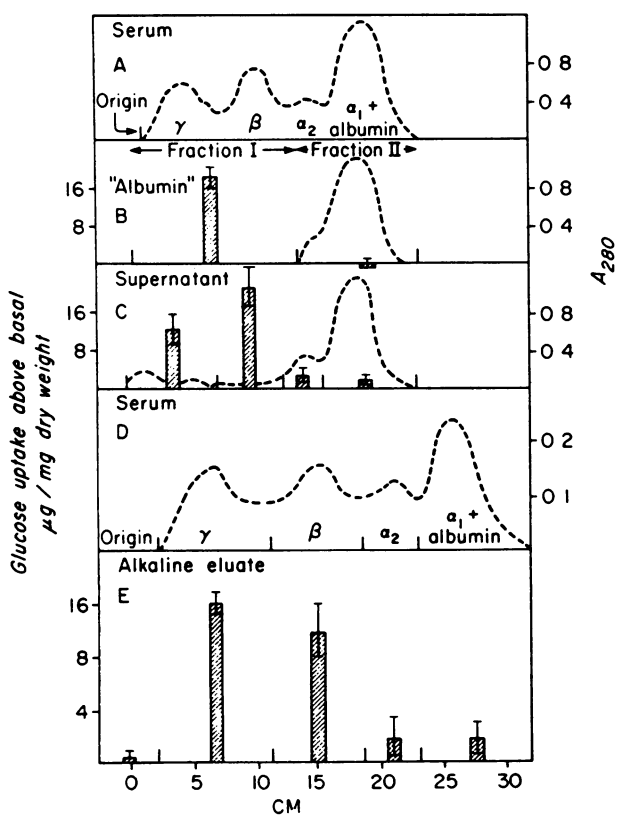

FIGURE 6 Electrophoretic mobility of ILA in "albumin," supernatant material, and alkaline eluate. "Albumin" (2 g/20 ml), supernatant material (0.5 g/20 ml), and alkaline eluate (8 mg) were submitted to zone electrophoresis under conditions described for serum (Fig. 1) and are shown as B, C, and E, respectively. In A, electrophoresis of serum (2 ml) in parallel with either "albumin" or supernatant material served as a marker for segmentation of the Pevikon block. In B, all of the electrophoretic fractions were combined into two pools, called Fractions I and II, as indicated by the short vertical lines. Fractions I and II were assayed by the rat hemidiaphragm response at 10 and 40 mg/ml, respectively. In C, all of the electrophoretic fractions were combined into four pools, as indicated by the short vertical lines. The four fraction pools were assayed at 5 mg/ml for the $alpha_2$ and 20 mg/ml for the $alpha_1$ globulin–albumin fraction pool. Bars represent the mean net glucose uptake for two experiments from separate serum pools and vertical lines indicate SEM. In D is shown the migration of serum proteins run in parallel with the alkaline eluate protein. In E, the electrophoretic fractions were combined into five pools, as indicated by the short vertical lines. The bars indicate the mean glucose uptake ± SD (three hemidiaphragms per determination) of the five fraction pools tested.

Electrophoretic mobility of ILA in "albumin," supernatant material and alkaline eluate protein. The migration of ILA in "albumin," supernatant material and alkaline eluate protein was determined by zone electrophoresis in Tris-EDTA-borate buffer, pH 9, with serum run in parallel as reference. As illustrated in Fig. 6, the ILA in all three serum protein fractions was distributed in zones corresponding to gamma and beta globulins. Pevikon electrophoresis of "albumin" and supernatant material resulted in separation of ILA from the major protein of these preparations. The heterogeneity of these extracted products is suggested by the presence of an alpha$_2$ migrating protein during electrophoresis of both "albumin" and supernatant material; whether this secondary peak consisted of albumin with slower migration or an alpha globulin was not ascertained. The electrophoretic mobility of the ILA in the alkaline eluate corroborates the observations of Antoniades, Huber, and Boshell (38). Because of the small amounts of protein used in the zone electrophoresis, no protein peaks could be measured from the block, however subsequent to electrophoresis of the alkaline eluate on cellulose acetate in 0.075 M barbital buffer, pH 8.6, protein was only detected in the alpha$_1$ globulin region. Thus from the migration of the ILA in the gamma-beta zone and the evidence that most of the protein was located in the alpha$_1$ globulin region, it would appear likely that higher specific activity for the ILA could be achieved.

Estimation of molecular weight of ILA by gel filtration. By means of gel filtration with Sephadex G-100 equilibrated in 0.15 M phosphate buffer, pH 7.4, the approximate molecular size of the ILA in unfractionated serum, gamma and beta globulin preparations, acid-ethanol extracts of serum, and alkaline eluate was determined. As illustrated in Fig. 7, proteins of known molecular weight were filtered to determine the volume of distribution from which distribution coefficients (K_d) were calculated.

Gel filtration of 20 ml of serum, concentrated to 5 ml before application to the column, resulted in an incomplete separation of two heterogeneous protein groups (Fig. 7). The first peak ($K_d = 0$), characterized by cellulose acetate electrophoresis, contained gamma and beta globulins and an alpha$_2$ migrating protein. The second peak ($K_d = 0.194$)

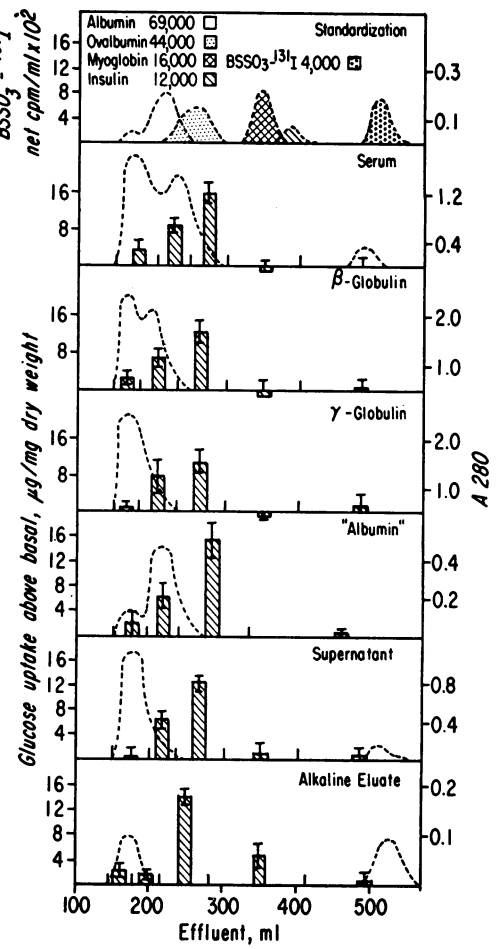

FIGURE 7 Distribution of ILA by gel filtration of serum, gamma, and beta globulin, acid-ethanol extracts of serum, and alkaline eluate protein. Ascending filtration was performed through a column (180 cm × 2 cm) of Sephadex G-100 in 0.15 M phosphate buffer, pH 7.4. Proteins of known molecular weight were separately filtered for standardization. Fractions from each gel filtration were arbitrarily combined into fraction pools as indicated by the short vertical lines. The fraction pools were assayed by the rat hemidiaphragm response. Bars represent mean net glucose uptake of each fraction pool for two experiments (three hemidiaphragms per experimental group) using separate serum pools, and vertical lines indicate SEM.

contained albumin and alpha and beta globulins. The smaller third peak ($K_d = 0.924$) was not characterized by electrophoresis. On bioassay of the filtration fractions of serum, the major peak of ILA was distributed between 200 and 300 ml of effluent, whereas crystalline insulin ($K_d = 0.722$) was eluted between 360 and 420 ml. By means of the immunoassay for insulin by the

double antibody technique (62), 18 μU of immunoreactive insulin (IRI) was detected in the effluent fraction between 300–400 ml, which corresponded to the distribution volume of crystalline insulin. IRI was not measurable in any of the other fractions.

Beta and gamma globulin preparations from zone electrophoresis of serum were also separately filtered. A biphasic protein distribution was obtained from the beta globulin filtration and possibly corresponded to high molecular weight globulins (greater than 100,000) and metal-binding globulins approximating 90,000 molecular weight ($K_d = 0.096$) (63). Filtration of gamma globulin produced a single peak excluded with the void volume and correlated with the generally accepted molecular weight of 160,000 for these proteins (63).

As shown in Fig. 7, the ILA in both the beta and gamma globulin fractions, like that in unfractionated serum, was distributed in an effluent volume of 230 to 300 ml, and partial separation from the major protein peaks was obtained. Similarly, the ILA in the acid-ethanol–extracted "albumin" and supernatant material was partially separated from albumin and distributed in the same effluent volume as ILA in unfractionated serum and the beta and gamma globulins from zone electrophoresis of serum.

Following gel filtration of alkaline eluate, two major protein peaks were observed. The first was excluded from the gel in the void volume corresponding to a substance of molecular weight of 100,000 or greater. The second peak was eluted with a coefficient of distribution ($K_d = 0.946$) of a substance of low molecular weight (less than 10,000). The major peak of ILA, assayed by the rat hemidiaphragm, was distributed in the effluent fraction between 200 and 300 ml corresponding to the behavior of the ILA in the other fractions.

In these studies, the eluates from the gel filtration column were pooled into five fractions, thus somewhat limiting the exact resolution of the elution pattern of ILA; however, since all of the fractions tested encompassed the elution behavior of compounds of molecular weight from above 100,000 to below 4000, it seems reasonable to conclude that most of the biological activity was distributed in a volume of 200–300 ml. By correlation with the distributions of the standard proteins, the K_d for the ILA in *all* of the preparations subjected to gel filtration probably ranged from 0.193 to 0.330. A mean K_d of 0.261 for the ILA in serum and fractionated products of serum agrees closely with the K_d of 0.266 for ovalbumin which has a molecular weight approximating 44,000, and therefore it seems likely that the molecular weight of ILA was in the general range of 40,000–50,000.

Effect of temperature and pH on alkaline eluate ILA. As shown in Table VIII, heating insulin or alkaline eluate ILA at 60° or 80°C for 5 min did not significantly alter the biological activity assayed by the rat hemidiaphragm. In Fig. 8 is illustrated the effect of pH on the biological activity of alkaline eluate ILA. It is apparent that the ILA was not affected by exposure to pH ranging from 2 to 10, however, after incubation by pH 12, virtually all biological activity disappeared. These findings are in agreement with those reported by Bürgi, Müller, Humbel, Labhart, and Froesch (30) who observed that a partially puri-

TABLE VIII

Effect of Heat on the Biological Activities of Insulin and Alkaline Eluate ILA

Experimental* group	Concentration	Glucose uptake‡ μg/mg dry wt		
		Control	5 min	5 min
		23°C	*60°C*	*80°C*
Buffer		17.30 ± 2.00	15.86 ± 1.64	16.88 ± 0.15
Insulin	1000 μU/ml	43.46 ± 1.76	43.20 ± 0.50	38.34 ± 1.34
Alkaline eluate protein	310 μg/ml	36.56 ± 1.80	36.74 ± 1.36	35.68 ± 0.98

* Experimental groups were prepared in Gey and Gey buffer with glucose and heated in a water bath. Bioassay was performed by the rat hemidiaphragm method after cooling and regassing.

‡ Each value represents mean glucose uptake of three hemidiaphragms ± SD.

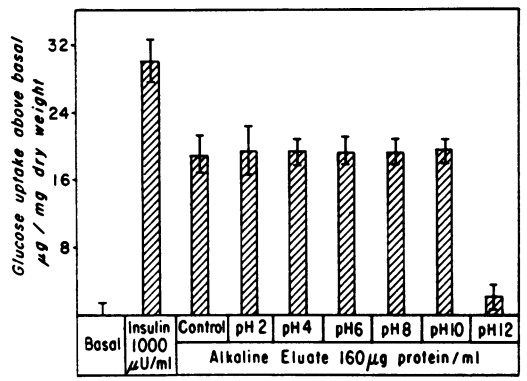

FIGURE 8 Effect of pH on biological activity of alkaline eluate ILA. Alkaline eluate (200 mg of salt), dissolved in 20 ml of deionized water, was divided into seven equal aliquots. After adjustment of pH with sulfuric acid or ammonium hydroxide, the solutions were left at 4°C for 4 hr. Dialysis was then performed against 100 volumes of Gey and Gey buffer with glucose added at 4°C in preparation for hemidiaphragm bioassay. The bars represent mean glucose uptake of three hemidiaphragms ± SD.

fied preparation of nonsuppressible ILA was both heat stable and denatured at alkaline pH.

DISCUSSION

In these studies, fractionation of serum protein was performed by (*1*) preparative zone electrophoresis, (*2*) acid-ethanol extraction of TCA protein precipitates, (*3*) isoelectric precipitation of albumin from acid-ethanol extracts, and (*4*) gel filtration. Analysis of the resulting serum protein fractions for ILA demonstrated the presence of a substance in serum with biological similarities to crystalline insulin when tested in vitro, but differring from insulin in physical and immunological properties.

It is established that crystalline insulin migrates similarly to alpha$_1$ globulins of serum in electrophoretic systems that utilize nonadsorbing media at pH values ranging from 7.4 to 8.6 (21) and in the present studies employing Pevikon at pH 9, this electrophoretic mobility for insulin-^{125}I added to serum was confirmed. However, after zone electrophoresis, ILA was detected in the gamma and beta globulin fractions, whereas no activity was found in the alpha$_1$ globulin–albumin fraction. Bolinger, van der Geld, and Willebrands, using the rat hemidiaphragm bioassay, have reported similar results (14). In contrast, by means of the more sensitive adipose tissue bioassay, ILA was found in gamma-beta globulin and alpha$_1$ globulin–albumin fractions, yet only the alpha$_1$ globulin–albumin migrating ILA has been shown to increase after glucose loading with no change in the level of gamma-beta migrating ILA (17, 19). Thus, serum immunoreactive insulin (IRI), endogenously released insulin in response to glucose and serum ILA migrating in the alpha$_1$ globulin region are presumably identical. Since the fasting serum level of IRI in normal subjects is under 50 μU/ml (4), which is below the limit of sensitivity of the rat hemidiaphragm bioassay, it is not surprising that significant levels of ILA were not detected in the alpha$_1$ globulin–albumin fraction.

By means of zone electrophoresis using paper (15), cellulose (12, 13), potato starch (14), and Pevikon (17–19), serum ILA has been shown to migrate in the gamma-beta globulin region, however, its nature has been controversial. Several investigators have suggested that the ILA in gamma-beta globulin preparations is, in fact, due to insulin bound to these proteins (13, 18, 20). Berson and Yalow have extensively studied this aspect and find no evidence for in vivo or in vitro binding of insulin to serum protein in noninsulin-treated subjects (21), and in the present experiments the failure of insulin antiserum to suppress the ILA of gamma and beta globulins appears to support their conclusions. However, partial-to-complete suppression of this ILA has been observed by others (13, 18). These discrepancies are not easily reconciled. If insulin is bound to gamma and beta-migrating serum proteins, it seems unusual that it is dissociated at pH 7.4 in 0.15 M phosphate buffer, as was observed during gel filtration of these proteins. Furthermore, the present observations suggest that the molecular weight of the active component of these electrophoretic preparations approximates 40,000–50,000, whereas both crystalline insulin and serum IRI appeared in a different fraction of lower molecular size.

In these studies, the only physicochemical property common to insulin and this serum insulin-like substance was solubility in acid-ethanol. Previously, serum ILA extracted into acid-ethanol has been attributed to endogenous insulin (22–24); however, the present studies indicate that this

concept is no longer tenable. Acid-ethanol extracts of serum do contain insulin at concentrations similar to those detected in serum by immunoassay (28), but bioassay of these extracts indicate that the major portion of ILA is unreactive with insulin specific antiserum, a finding which in conjunction with its behavior during electrophoresis and gel filtration supports the conclusion that this molecule is not crystalline insulin. Because more ILA is detected in serum after acid-ethanol extraction, it has been suggested that the ILA is either "released" or modified by this procedure, so that it is readily measured by in vitro bioassays (22, 23, 64). However, at present, unequivocal data are not available to support this assumption. In the experiments presented in this paper, the acid-ethanol–extracted ILA, unreactive with insulin antibody, behaved identical to that found in unaltered serum as shown by electrophoretic mobility and gel filtration.

Ion exchange chromatographic techniques have not been extensively employed in the study of insulin or insulin-like activity in serum. Power, Reyes-Leal, and Conn have shown that the ILA in serum globulin fractions obtained by ammonium sulfate precipitation can be eluted from DEAE-cellulose under conditions of decreasing pH and increasing ionic strength of the buffering medium (65). It has been assumed that this ILA represents insulin. The present studies of the behavior of ILA in both serum and acid-ethanol extracts of serum demonstrate that, under conditions of stepwise elution, ILA can be separated from beta and gamma globulins but not from albumin. That this ILA is not equatable with insulin is supported by the inability of insulin specific antisera to neutralize its biological activity and the distinctly different chromatographc properties of both insulin-^{131}I and crystalline insulin. Although ILA could not be separated from albumin on DEAE-cellulose, the fact that the ILA in both serum and acid-ethanol extracts of serum migrates on zone electrophoresis distinct from albumin indicates that ILA is not a property of the albumin molecule.

The present investigations of the distribution of ILA on the cation exchange resin Dowex 50 confirm the results of Antoniades and coworkers (32–34, 38). Dowex 50 × − 8, 25–50 mesh size, was originally used as described by Antoniades (60), but was displaced in favor of 100–200 mesh resin because of apparent incomplete adsorption of ILA. In these studies, a resin to serum ratio of 1 : 1 was found to result in maximal retention of ILA. A previous report on the behavior of crystalline insulin under various conditions of chromatography on Dowex emphasizes the importance of pH (61). In phosphate buffers at pH below 6.8, insulin is adsorbed to the resin, which probably reflects diminished solubility below pH_I 7.0 (66) as insulin is cationic only below pH 5.3 (66). Since serum approximates pH 8 when exposed to a normal laboratory environment, immunoassayable insulin would be expected to pass through the resin and this has been confirmed for both unlabelled and radiolabelled insulin (33, 61). The failure to find immunoreactive insulin in the resin-adsorbed protein fraction (34, 38) or to inhibit the ILA of this fraction with insulin antiserum (38) (both of which are confirmed by the present studies) conforms to the expected anionic behavior of insulin at this pH. Because of the adsorption of ILA on the resin under conditions which distinguish it from insulin, Antoniades and colleagues have referred to this ILA as "bound insulin." It was originally proposed that insulin was bound to a basic protein that imparted a net cationic charge to the molecular complex; however, since attempts to dissociate immunoassayable insulin from the resin-adsorbed insulin-like substance have been unsuccessful for the most part (36–38), it has been suggested that "bound insulin" may represent either a combination of insulin molecules associated with other serum macromolecules (38) or possibly a substance structurally distinct from insulin, the physiological significance of which is not understood at present (28–31). From our results, it is difficult to make definite conclusions regarding the ionic nature of the insulin-like substances in human serum since studies of the fundamental mechanism of adsorption of proteins and other macromolecules onto ion exchange surfaces are lacking (67). Most certainly, factors in addition to molecular charge play significant roles in determining the chromatographic behavior of complex molecules. This would seem particularly the case with serum ILA since it would appear to behave as a cation on Dowex 50; yet on DEAE-cellulose, it does not exhibit the characteristics of a cation.

Although the present studies corroborate the

findings of Antoniades and coworkers that ILA is adsorbed onto Dowex, a perplexing discrepancy exists in the results obtained for the biological activity of this ILA on muscle. Based on data derived from the rat hemidiaphragm bioassay of serum and "bound insulin" preparations, with and without the presence of an extract of adipose tissue (ATE), it has been concluded that "bound insulin" is inactive on muscle unless dissociated or activated by a low molecular substance present in adipose tissue extracts (60, 68). In our hands, however, the hemidiaphragm bioassay of the resin-adsorbed protein ("bound insulin") consistently yielded considerable biological activity in the absence of ATE. Certainly, variations in the method of chromatography and in the bioassay procedure may account for this difference in part; since comparative studies with and without ATE were not done, this question remains unanswered. It should be noted, however, that the specific activities of several eluate fractions bioassayed without ATE moderately exceeded values recently reported by others employing ATE (34, 41, 60). Fractionated human serum ILA prepared by a variety of other methods was also biologically active on muscle in vitro (38). In addition, "bound insulin" preparations injected in vivo appear to promote glycogen synthesis in muscle (34). It appears, therefore, that the concept of biological inactivity of this insulin-like substance on muscle must be modified and the problems of hemidiaphragm bioasay of undiluted serum closely examined.

In most of the studies of serum ILA, the parameters of glucose uptake in isolated muscle or glucose oxidation in isolated adipose tissue have been used. The specificity of these effects has been questioned because glucose utilization in isolated tissues has been stimulated by environmental alterations that include hyperosmolarity (69) and anaerobiasis (70), as well as uncoupling agents (70) and certain amino acids and hormones (3). In the present experiments, in addition to stimulation of glucose uptake, the ILA in the gamma-beta migrating zone from serum, "albumin," supernatant material, and alkaline eluate protein enhanced glycogen synthesis in the rat hemidiaphragm, an effect which has not been observed with the above environmental factors and which has been specifically attributed to insulin (71, 72). While some investigators have demonstrated both stimulation of glucose uptake and glycogen synthesis in muscle by chymotrypsin and trypsin (73), others have failed to confirm this finding (74). That chymotrypsin or trypsin can be equated with serum ILA seems unlikely, since the concentration of chymotryptic activity in serum is below that required for an in vitro effect (75, 76). Moreover, the molecular weights of pancreatic preparations of chymotrypsin (25,000 mol wt) and trypsin (20,000 mol wt) (77) differ from that observed for ILA.

Previous studies of some of the physicochemical properties of "atypical" insulin, "bound" insulin, and NSILA are in general agreement. Samaan, Fraser, and Dempster, by dialysis, have estimated the molecular size of "atypical" insulin at greater than 30,000 (9). "Bound" insulin has been reported to migrate in the gamma and beta globulin region on zone electrophoresis and from its behavior on Sephadex G-100 gel filtration at pH 8.0, Antoniades and colleagues have concluded that "bound" insulin has a molecular weight between 60,000 and 100,000 (38). Bürgi and associates have reported beta and alpha$_2$ globulin electrophoretic migration of a highly purified preparation of NSILA (30). On the basis of Sephadex G-200 filtration of pH 7.2, the latter workers have also estimated the molecular weight of the NSILA of serum to range from 70,000 to 150,000. However, when a highly purified preparation of serum NSILA was subjected to gel filtration through G-75, a broad indefinite peak of ILA was obtained, which suggests the possibility of multiple polymeric forms of this insulin-like substance. Furthermore, the highly purified serum NSILA appeared to "dissociate" into compounds with a molecular weight approximating 6000 to 10,000 when subjected to gel filtration in 5 M acetic acid (30). In the present studies, in which the unfractionated serum, gamma and beta globulin fractions from zone electrophoresis, acid-ethanol extracts, and alkaline eluate protein were subjected to gel filtration, the major peak of ILA, not suppressed by insulin antiserum, was eluted in a volume corresponding to the behavior of a substance of molecular weight of approximately 40,000–50,000. Although precise resolution was not obtained by definition of the volumes pooled for bioassay, the general symmetry of distribution of

ILA suggests that the filtration pattern represented a single peak of ILA with a molecular size approximating that of ovalbumin. It is conceivable that the discrepancy in the apparent molecular weight for ILA in neutral buffers between these studies and those of Bürgi and coworkers (30) and Antoniades and colleagues (38) may be due to the improved resolution provided by the increased dimensions of the column and the choice of Sephadex G-100 in these experiments.

The importance of pH in "activating" alkaline eluate ILA has previously been reported (33, 78). Apparently, isoelectric precipitation of protein in the alkaline eluate was observed after pH adjustment to 9.8 to 10. The supernatant resulting from this procedure contained biological activity on the rat hemidiaphragm without ATE. In the present studies, pH adjustment of the Dowex alkaline eluate over a wide range (from pH 2 to 12) did not result in observable precipitation of protein nor increase in biological activity at pH 10. Inactivation of biological activity at pH 12, however, did provide correlation with the reports of alkaline instability of a partially purified preparation of serum nonsuppressible ILA (25). In addition, this preparation of ILA was heat stable at 70° and 80°C (30), a finding in agreement with the observed heat stability for Dowex-adsorbed ILA in the present studies.

On the basis of in vitro biological activity, lack of suppression of activity by insulin antiserum, behavior on anion and cation exchange resin, electrophoretic mobility, and molecular weight, it seems probable that "bound insulin," "total serum insulin activity," "atypical insulin" and "nonsuppressible insulin-like activity" are identical. Because the physical properties of insulin and ILA are markedly different, a structural resemblance to insulin remains conjectural. If this ILA consists of insulin complexed to a macromolecule in serum as has been suggested (33), it is necessary to invoke a strong covalent bond between insulin and its "carrier," since the procedure of acid-ethanol extraction would be expected to dissociate molecules aggregated by weaker forces such as hydrogen bonding or Van der Waal's forces. Because there is as yet no convincing evidence that ILA is related structurally to insulin, the term "nonsuppressible insulin-like activity" (NSILA), proposed by Froesch, Bürgi, Ramseier, Bally, and Labhart (29), would seem to us to be the most appropriate description of this substance.

Thus, from the studies reported herein, it seems justified to conclude that both insulin and a single molecular species distinct from insulin by physicochemcial criteria contribute to the measurement of ILA in in vitro bioassay systems.

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