Characteristics of Nonsuppressible Insulin-like Activity in Fasting Human Serum

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SUMMARY

Insulin-like activity (ILA) and nonsuppressible insulin-like activity (NSILA) of fasting normal human serum have been studied following fractionation by cation resin chromatography, or extraction with acid-ethanol or ethanol. Studies of solubility, heat stability and immunosuppressibility suggest three different fractions, none of which is immunoassayable insulin. The total activity of the fractions was usually several times that of the original serum.

"Bound" NSILA, which binds to a cation resin column, appears to be a smaller molecule than "free insulin" NSILA found in the column effluent. Our studies suggest that the column effluent NSILA is converted to the smaller "bound" NSILA by acid-ethanol extraction. Although the column effluent NSILA is partially suppressible with guinea pig antiserum, only a trace amount of immunoassayable insulin was found in this fraction. DIABETES *21*:271-79, May, 1972.

The major portion of insulin-like activity, ILA, in fasting serum, differs from crystalline insulin in molecular size, electrophoretic mobility and immunosuppressibility. It has therefore been designated nonsuppressible insulin-like activity, NSILA,[1] or atypical insulin.[2]

Several authors have suggested that NSILA is a combination of more than one form of activity. Jakob et al.[3] found the acid ethanol insoluble fraction contained nearly 90 per cent of serum NSILA determined by fat pad assay. Molecular weight was estimated at 100,000-150,000; it was heat labile at 80° C.; would not bind to a cation resin column; and could not be converted to a smaller molecular size by acetic acid-NaCl chromatography. The acid ethanol soluble fraction contained less than 10 per cent of the total activity. It was heat stable at 80° C. for fifteen minutes and was bound on a cation resin column. Sephadex chromatography in acetic acid-NaCl reduced it from a molecular size of 20,000-80,000 to 6,000-10,000.

ILA which is removed from serum by adsorption on a cation resin column has been termed "bound" insulin by Antoniades.[4] It has been shown that the acid ethanol soluble fraction of NSILA adsorbs to the cation resin as does "bound" insulin of Antoniades and therefore is assumed to be the same.[3,5]

From the results of serum electrophoresis, acid ethanol extraction, DEAE cellulose fractionation, and cation resin chromatography it was concluded that "bound" insulin, "atypical" insulin and NSILA are the same substance.[5] These authors obtained two fractions of NSILA after passage of serum through a DEAE cellulose column. However, only the smaller molecular size fraction was present in the acid ethanol extract. This fraction was found to be similar to "bound" insulin. Antoniades et al. have termed ILA in serum not bound to the cation resin column as "free" insulin,[4] and have related it to crystalline insulin.[6] However, suppression of this fraction by antisera has not been demonstrated in fasting serum. Gjedde[7] obtained three peaks of ILA in serum fractions from Sephadex G-200 corresponding to macroglobulin, albumin and polypeptides. Labeled insulin added to serum was always associated only with the peptide peak.

The diverse nature of ILA fractions is further complicated by activation and inhibition which have been reviewed by Batchelor.[8] In addition to the increased activity with dilution, many investigators have found greatly increased ILA following acid-ethanol extraction.[9-12] Increased activity of undiluted serum after passage through a cation resin column has been a consistent finding in our laboratory and has been noted by others.[3]

Reports of the immunosuppressibility of ILA following fractionation or activation are inconsistent. Shaw and Shuey[13] reported "bound" ILA to be immunosuppressible after treatment with adipose tissue extract.

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Antoniades et al.[14] showed partial suppression of acid alcohol treated "bound" insulin but the kinetics of antisera activity were different than with crystalline insulin. Gjedde[15] found the two larger molecular size fractions of ILA were not immunosuppressible until after dialysis against calcium chloride and weak alkali. Most of the studies in which partial immunosuppression of ILA has been reported have utilized the fat pad.[2,16,17] Contrary to the above findings, Kajinuma and coworkers,[18] also using the fat pad assay, did not observe any increase in immunosuppressible ILA after extraction of serum with acid-ethanol-NaCl.

As previously reported,[19] we have not been able to confirm the activation of "bound" insulin by any of the methods proposed by Antoniades. We have greatly increased the activity of the original serum both by column fractionation and extraction. Further investigations have suggested the presence of three forms of NSILA in fasting serum. The term NSILA as used in this paper refers to all ILA which is not suppressed by insulin antisera.

MATERIALS AND METHODS

Cation resin column fractionation of ILA

Fresh normal human serum was obtained from blood of fasting individuals. One liter of serum, containing a tracer amount of radioiodinated human serum albumin, was passed through an equal volume of Dowex 50-X8, 100-200 mesh, (Na^+ cycled) resin in a 4.6 cm. diameter column at a flow rate of approximately 10 ml. per minute. After the void volume, the effluent serum was collected and corrected for dilution by measurement of radioactivity. The column was then washed with two volumes of 0.15 M NaCl at the same rate. The resin was eluted at 4° C. with one volume of 0.02 M NH_4OH over a one to two minute period. The eluate was neutralized with 0.2 N H_2SO_4 by automatic titration. The lyophilized eluate yielded about 4 mg. of powder per milliliter of serum, consisting mainly of sodium and ammonium salts. This material is referred to as alkaline eluate. Prior to assay, the powder was made to the original serum volume in water containing human serum albumin, 0.5 mg./ml., and dialyzed for twenty-four hours at 4° C. against two changes of Gey and Gey[20] bicarbonate buffer containing glucose, 3.0 mg./ml. The original and effluent serum fractions were similarly dialyzed prior to assay. Before being used, the dialysis tubing was boiled for approximately eight hours with several changes of water to remove volatile and soluble sulfur compounds. In order to evaluate possible column effects, in one study Gey and Gey buffer was passed through a column, eluted, and assayed in a manner identical to serum.

Multiple column fractionation

Serum was passed successively through three separate columns having resin-to-serum volume ratios of 3.7 to 1. The serum was collected with very little dilution and the resin volume of the succeeding column was made equal to the effluent serum volume. An aliquot of the column effluent serum was saved for comparative study. The first column was eluted twice with NH_4OH and saline. The successive columns were eluted once.

Acid ethanol fractionation

Original serum and effluent serum from the cation resin column were extracted by the method of Jakob.[3] The soluble fraction was lyophilized and then made to volume with water prior to dialysis. The insoluble fraction was evaporated to dryness and suspended in water prior to dialysis.

Ethanol fractionation

Normal and column effluent sera were subjected to extraction in 70 per cent ethanol. Nine volumes of 77 per cent ethanol were added to one volume of serum. The insoluble fraction was made to the original serum volume in Gey and Gey buffer and re-extracted in another nine volumes of ethanol. A total of three extractions was done. The soluble and insoluble fractions were processed as in the previous study.

The effect of acid upon ILA and NSILA

In addition, to determine the optimum pH for activation, serum aliquots were treated at room temperature for two hours at a pH ranging from 2 to 7.6 and neutralized by dialysis which required two changes of Gey and Gey buffer containing glucose, 3 mg. per milliliter. Original serum, column effluent and alkaline eluate were each subjected to hydrochloric acid at a pH of 3.5 for a period of two hours.

Heat stability

Original serum, effluent serum, and alkaline eluate were heated at 80° C. for forty-five minutes at neutral pH. Other samples were adjusted to pH of 3.5 with HCl and heated at 80° C. for forty-five minutes and neutralized by dialysis as above.

Rat diaphragm bioassay procedure

Control incubation media consisted of Gey and Gey bicarbonate buffer with glucose, 3 mg. per milliliter, and human serum albumin, 0.5 mg. per milliliter. The sample to be assayed was dialyzed against Gey and Gey plus glucose as previously described. Uniformly labeled C-14-glucose, 0.5 μc. per milliliter, was added to both control and experimental assay material.

Sprague-Dawley rats weighing between 90 and 120

gm. were maintained on Purina Rat Chow under well-regulated environmental conditions. Animals matched for weight were fasted for twenty-four hours prior to each study. They were sacrificed by decapitation and exsanguinated to prevent blood from coming in contact with the hemidiaphragms. The right and left hemidiaphragm from each animal was removed and individually transferred to freshly prepared ice-cold Gey and Gey bicarbonate buffer saturated with 95 per cent oxygen and 5 per cent carbon dioxide. After approximately twenty-five minutes, which was required for removal of four sets, each set of hemidiaphragms was removed from the buffer, gently blotted and one hemidiaphragm was placed in a 25 ml. Erlenmeyer flask containing the assay sample and the other placed in a flask containing control incubation medium. To compensate for any difference between the two sides, the left and right hemidiaphragm were alternated between assay and control media. The incubation vessels were gassed for five minutes with 95 per cent oxygen and 5 per cent carbon dioxide while shaking in a 37° C. water bath at 120 oscillations per minute. The vessels were then capped and incubated for an additional eighty-five minutes.

The hemidiaphragms were then removed from the incubation vessels, rinsed in distilled water, blotted dry between sheets of Whatman filter paper and weighed. The amount of glucose incorporated into glycogen by the hemidiaphragms was determined by the method of Rafaelsen et al.[21] The final precipitates were dissolved in 1 ml. distilled H_2O and mixed. Aliquots, 0.2 ml., from each tube were counted in a Packard Tri-Carb Liquid Scintillation Counter, Model 3380, to a counting error of less than 2 per cent. Values were calculated as micrograms glycogen produced per 10 mg. wet tissue. Results are expressed as difference from control hemidiaphragm.

In order to make a valid comparison of serum treatments, the ILA and NSILA of control, acid ethanol soluble, acid ethanol insoluble and acid treated original serum were determined simultaneously. Eight determinations were obtained for each study which included one of each of the above. Four or more such studies were done for any one set of values. The effluent serum and alkaline eluate were studied in a similar manner.

Immunosuppressibility of ILA

Antisera to bovine insulin was produced in guinea pigs by the method of Wright, Makula, and Posey.[22] An antiserum dilution of 1:100 in the rat hemidiaphragm assay system completely suppressed the activity of 1,000 μU. per milliliter of insulin. Fractions were studied with and without addition of antisera.

Radioimmunoassay of insulin

Fractions were studied for immunoassayable insulin using three methods of separation of the antibody bound and free insulin. These included paper chromatography,[23] double antibody,[24] and ethanol precipitation.

RESULTS

Serum ILA fractions obtained from the cation resin column

Since the activity of the effluent serum and alkaline eluate each equal that of the original serum, the total activity is doubled in a single passage through a cation resin column (table 1). The additional activity was not considered to originate from the column since the passage of buffer followed by alkaline elution resulted in no activity. The activity of the alkaline eluate was obtained without any of the methods of activation proposed by Antoniades[14,25] and Gundersen and Lin.[26]

TABLE 1

Recovery of ILA from cation resin column

Fraction	Immunoassayable insulin μU./ml.	Glycogen production μg./10 mg. wet tissue ILA	NSILA
Gey and Gey buffer-alkaline eluate		(8) —1.0 ± 0.7*	
Original serum	(6) 11.4 ± 3.0	(14) 13.1 ± 2.3	(14) 15.5 ± 2.4
Effluent serum	(6) 16.4 ± 3.4	(14) 13.7 ± 1.4	(14) 7.6 ± 1.7†
Alkaline eluate	(6) —0.4 ± 2.1	(8) 10.9 ± 2.8	(8) 13.7 ± 1.3
Insulin standards	100 μU.	(30) 7.6 ± 0.5	
	1,000 μU.	(5) 38.9 ± 3.3	(5) 0.1 ± 1.1†

( ) Number of determinations
* Mean ± SEM
† NSILA differs from ILA, $p < 0.01$

TABLE 2

Recovery of ILA from three successive columns

Sample	Glycogen production-μg./10 mg. wet tissue		
Original serum	(9) 13.5 ± 2.0*		
Column fractions	First column	Second column	Third column
Effluent serum	(23) 18.6 ± 1.7		(4) 13.8 ± 2.0
Alkaline eluates			
First elution	(4) 24.0 ± 3.8	(4) 14.1 ± 3.9	(4) 4.9 ± 1.5
Second elution	(4) 13.1 ± 3.2		

( ) Number of determinations
* Mean ± SEM

The apparent lack of an immunosuppressible insulin in the original serum reflects the low level of immunoreactive insulin in the fasting state. The effluent serum ILA was partially, and consistently, suppressible. However, there was essentially no immunoassayable insulin detectable in this fraction. The ILA of the alkaline eluate is nonsuppressible and nonimmunoassayable. Insulin standards are shown for comparison only. They were not passed through the resin column.

Multiple column fraction studies

Serum ILA recovered from a single column is further amplified in multiple column studies as shown in table 2. Since the second alkaline elution of the first column yielded additional ILA, additional elutions could potentially increase the activity. The ILA of effluent serum remains essentially unchanged after passage through three columns.

Acid ethanol fractionation

The results of acid ethanol fractionation of original and effluent serum are shown in table 3. The ILA in the soluble fraction exceeds that of the original serum and is nonsuppressible. The activity of the insoluble fraction was equal to the original and was also nonsuppressible. The soluble and insoluble fractions of the effluent serum are each equal to the effluent serum activity. Although the data is not statistically significant, the soluble fraction appears to be partially suppressible. The insoluble fraction remains nonsuppressible.

Ethanol fractionation of NSILA

The results of multiple ethanol extraction of serum are shown in table 4. The sum of the soluble and insoluble fraction of the first extraction exceeded the original serum activity. Repeated extraction of the insoluble fraction yielded additional soluble activity with resultant decrease in the insoluble fraction. With the ten volumes of solutions used, it is unlikely that the subsequent recovery of soluble activity represented material trapped in the precipitate. These results suggest that a portion of the insoluble activity is converted to soluble activity with each subsequent extraction. As with multiple column fractionation, the total NSILA obtained by multiple extractions greatly exceeded the original serum activity.

The effect of acid upon ILA

The optimum pH for activation of serum ILA was found to be approximately 3.5 as shown in table 5. The

TABLE 3

Acid-ethanol extraction
original and column effluent sera

Fraction	Glycogen production μg./10 mg. wet tissue ILA	NSILA
Original serum	(14) 13.1 ± 2.3*	(14) 15.5 ± 2.4
Soluble	(12) 28.2 ± 2.1	(12) 23.9 ± 2.8
Insoluble	(12) 13.7 ± 2.2	(12) 11.9 ± 1.4
Effluent serum	(14) 13.7 ± 1.4	(14) 7.6 ± 1.7†
Soluble	(10) 15.4 ± 2.8	(10) 8.8 ± 2.0‡
Insoluble	(10) 15.2 ± 1.8	(10) 11.2 ± 1.8
Insulin (1,000 μU.)	(6) 37.9 ± 4.1	(6) 1.8 ± 0.5

( ) Number of determinations
* Mean ± SEM
†, $p < 0.01$; ‡, $p < 0.08$ that NSILA differs from ILA

TABLE 4

Multiple ethanol extraction* of normal serum

Treatment	NSILA Glycogen production μg./10 mg. wet tissue Soluble		Insoluble	
Original serum	(8)	9.9 ± 1.5†		
First extraction	(4)	7.9 ± 1.0	(4)	5.1 ± 0.7
Second extraction	(4)	5.1 ± 1.9	(4)	2.7 ± 2.0
Third extraction	(4)	5.9 ± 1.4	(4)	1.2 ± 1.0

( ) Number of determinations
* Each extraction with ten volumes of 66 per cent ethanol
† Mean ± SEM

effect of acid incubation upon the activity of the original serum, column effluent serum and alkaline eluate is shown in table 6. Although acid increased the ILA and NSILA of the original serum, no effect was noted with either the effluent serum or the alkaline eluate. All acid-treated fractions remained nonsuppressible.

TABLE 5

Optimum pH for acid activation of normal human serum

pH	Glycogen production μg./10 mg. wet tissue
7.6	(8) 8.3 ± 1.4*
5.0	(4) 11.5 ± 2.0
4.0	(12) 17.2 ± 1.7
3.5	(8) 19.0 ± 2.1
3.0	(12) 17.2 ± 1.9
2.0	(4) 10.4 ± 3.2

( ) Number of determinations
* Mean ± SEM

Heat stability

When the original or effluent serum was heated at neutral pH the ILA is nearly completely lost as shown in table 7. Upon heating, a thick precipitate formed which was included in the assay sample. The alkaline eluate did not form any precipitate and retained all its activity after heating. If the original or effluent serum was heated at pH 3.5 very little protein precipitation occurred and there was little loss of activity (table 7). The neutral and acid-treated sera were from different lots so the apparent absence of acid activation may be on this basis. In this study, there was no comparison of control and acid-treated serum without heating.

The acid ethanol soluble fraction of original serum, similar to the column alkaline eluate, is heat stable at neutral pH. The acid ethanol insoluble fraction of the original serum as well as both the acid ethanol soluble and insoluble fractions of effluent serum were found heat labile although no visible precipitate formed. This suggests that the precipitate was not necessary for heat inactivation. If heated at acid pH the insoluble fraction of original serum retains its activity as does the original and effluent serum.

DISCUSSION

In this study, three forms of NSILA have been

TABLE 6

Effect of acid treatment upon ILA and NSILA

Sample	Glycogen production μg./10 mg. wet tissue ILA		NSILA	
Original serum				
Control	(14)	13.1 ± 2.3*	(14)	15.5 ± 2.4
Acid treated	(8)	32.1 ± 3.7†	(8)	31.8 ± 2.7†
Effluent serum				
Control	(14)	15.2 ± 1.7	(14)	8.2 ± 1.8‡
Acid treated	(8)	11.9 ± 1.2	(8)	11.8 ± 0.7
Alkaline eluate				
Control	(8)	10.9 ± 2.8	(8)	13.7 ± 1.3
Acid treated	(8)	11.5 ± 2.1	(8)	13.0 ± 2.2

( ) Number of determinations
* Mean ± SEM
† Acid treated differs from untreated, $p < 0.001$
‡ NSILA differs from ILA, $p < 0.005$

TABLE 7

Effect of pH upon heat stability of ILA

Fraction	Glycogen production μg./10 mg. wet tissue	
	Control	Heated 80° C. for 45 min.
	Neutral pH	
Original serum	(4) 16.2 ± 2.4*	(4) 1.5 ± 1.8†
Acid ethanol soluble	(6) 30.1 ± 2.6	(6) 29.3 ± 1.3
Acid ethanol insoluble	(4) 13.7 ± 4.7	(4) 0.2 ± 1.9†
Effluent serum	(4) 15.2 ± 1.7	(4) 3.4 ± 1.6‡
Acid ethanol soluble	(4) 13.5 ± 2.2	(4)—0.3 ± 1.7†
Acid ethanol insoluble	(4) 11.6 ± 3.8	(4) 3.5 ± 1.9
Alkaline eluate	(4) 16.9 ± 1.7	(4) 14.6 ± 2.1
	Acid pH 3.5	
Original serum	(4) 13.1 ± 2.5	(4) 12.6 ± 2.6
Acid ethanol insoluble	(4) 13.7 ± 4.7	(4) 10.0 ± 2.2
Effluent serum	(4) 17.5 ± 2.4	(4) 17.9 ± 3.1

( ) Number of determinations
* Mean ± SEM
†, $p < 0.01$; ‡, $p < 0.02$ that heated differ from control samples.

demonstrated in fasting normal human serum. The original serum NSILA and its acid ethanol insoluble fraction, as well as the effluent serum and its acid ethanol soluble and insoluble fractions from a cation resin column are all mostly heat labile when heated at neutral pH. Heating at pH 3.5, which prevents protein precipitation, results in no loss of activity. The ethanol and acid-ethanol soluble fraction of the original serum and the alkaline eluate from the cation resin column are heat stable at neutral pH. In this regard these fractions differ from original serum activity although Poffenbarger and co-workers[5] found the unaltered serum and the acid ethanol extracted NSILA behaved identically by electrophoretic mobility and gel filtration.

Using the rat diaphragm bioassay, we found the acid ethanol soluble fraction contained two thirds of the total activity. Jakob et al.[3] found virtually all the NSILA in the insoluble fraction using the fat pad bioassay. Multiple ethanol extractions, table 4, suggest that much of the insoluble fraction may be slowly converted to a soluble form. These studies suggest that the acid-ethanol soluble fraction consists of NSILA from the original serum reduced to a smaller molecule which is more stable.

After passage of serum through a cation resin column, the column effluent serum contains NSILA which, like the original serum, is mostly heat labile at neutral pH and contains acid ethanol soluble and insoluble fractions. The activity of effluent serum is equal to or greater than the original serum. The activity which does not bind to the column has been labeled "free" insulin by Antoniades[14] and is being equated to crystalline pancreatic insulin.[6] Following a glucose load, and in pancreatic vein serum, immunoreactive insulin is present in significant concentration and will appear in the effluent serum. However, in fasting serum, the large amount of activity detected is nonsuppressible and contains no appreciable immunoreactive insulin. There are at least two forms of ILA which do not bind to the cation resin column; one is crystalline insulin, the other is NSILA. Therefore, the use of the term "free" insulin and the suggestion that it is crystalline insulin without showing immunoreactive properties seems unjustified.

We have been unable to duplicate the results of Poffenbarger et al.[5] in which the column effluent serum contained little activity. In our hands, the column effluent serum always contains NSILA equal to or greater than the original serum even with a column bed volume three to five times the serum volume.

The fraction of NSILA bound to the cation resin column which is eluted with alkali, is heat stable at neutral pH and is completely soluble in ethanol or acid ethanol. As previously reported[19] we find that the eluate from the cation resin column, which Antoniades has labeled "bound insulin," possesses activity in the rat diaphragm bioassay without prior activation by any of the methods proposed by Antoniades et al.[14,25] and Gundersen and Lin.[26] We found no increase in ILA after any of the proposed methods of activation. Poffenbarger and co-workers[5] recorded similar ILA in the alkaline eluate without activation. In the majority of

our studies the activity of the column eluate was greater than the original serum.

The quantity of alkaline eluate or "bound insulin" activity which is obtained from a given serum can be increased many times by the use of multiple columns and multiple alkaline extractions of a column (table 2). One might therefore question the validity of any quantitative studies regarding serum concentrations of "bound insulin" in relation to a glucose load.

The theory of "bound" and "free" insulin as proposed by Antoniades, appears especially open to criticism from the above studies. He proposes that "bound insulin" is a large molecule which is converted to a smaller, more active "free" insulin, the "bound insulin" being in the alkaline eluate and the "free" in the column effluent serum. Since the alkaline eluate or "bound" fraction appears to have soluble characteristics identical to the acid ethanol soluble fraction, it would appear that what is being called "bound" insulin is a smaller more soluble molecule than the "free" insulin found in the column effluent. This is in agreement with the studies of Jakob et al.[3] who found all of the low molecular weight NSILA of serum in the acid ethanol soluble fraction. Since our studies with multiple columns and multiple ethanol extractions both suggest that the majority of the NSILA can be converted to the "cation column bound" or ethanol soluble fraction, it would seem more logical to suggest that the column effluent NSILA or "free insulin" is converted to the cation resin bound NSILA or "bound insulin."

Although the original serum and column effluent NSILA both appear to be large size particles which are heat labile, the effluent NSILA differs from the original serum in that it lacks the property of acid activation (table 6). In addition, we have isolated a specific inhibitor which affects only the activity of the effluent serum and the acid ethanol insoluble NSILA. These results suggest that in addition to pancreatic insulin, serum may contain three forms of NSILA. These would include: 1) the acid ethanol soluble, heat stable NSILA of original serum which probably includes the alkaline eluate from the cation resin column; 2) the acid ethanol insoluble, heat labile NSILA of original serum, and 3) the column effluent acid ethanol soluble activity which is heat labile, not activated by acid and partially suppressed by antiserum. The characteristics of each fraction are summarized in figure 1.

The use of column fractionation or electrophoresis to estimate particle size has been omitted from these studies since the published literature yields confusing results. NSILA which is ethanol extracted with albumin migrates with beta and gamma globulin on electrophoresis. The separation of beta and gamma globulins on Sephadex appears too crude to fractionate NSILA. Atypi-

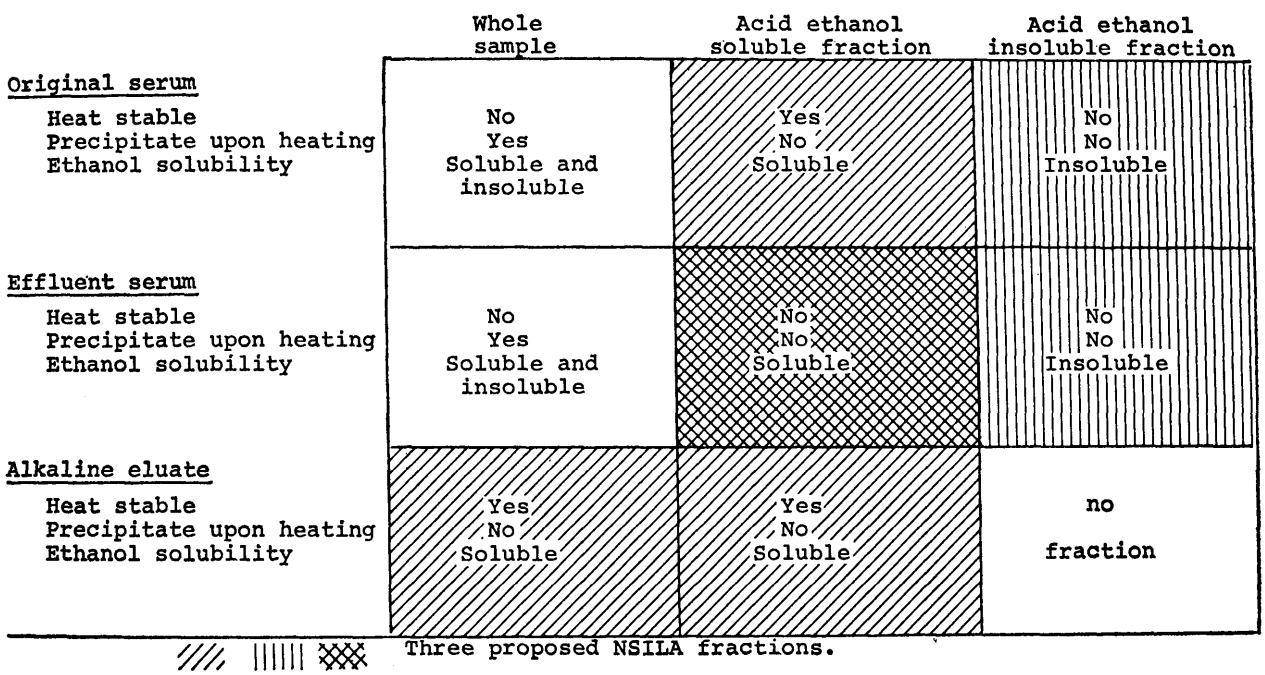

FIG. 1. Physical characteristics of ILA fractions.

cal insulin molecular weight has been estimated at 30,000,[2] "bound" insulin 60,000 to 100,000,[14] NSILA at 70,000 to 150,000[1] and 40,000 to 50,000,[5] with the suggestion that they are all the same substance.

The enhancement of NSILA demonstrated by column fractionation, ethanol extraction, and acid incubation may be due to separation or destruction of inhibitors or conversion to a more active molecule. The results are not due to an artifact from the column since no activity was obtained from a buffer treated column. The optimum pH of 3.5 for activation coincides with the optimum for alpha$_1$ anti-trypsin inactivation.[27] Two studies suggest that trypsin does possess insulin-like activity.[28,29]

The finding that none of the NSILA fractions are destroyed when heated at pH 3.5 lends support to the interpretation that the fractions vary in particle size. In those studies in which heating produced a precipitate the NSILA was lost. However, as stated in the text, three alcohol extracted fractions, in which no precipitate was formed, were also found to be heat labile.

We are unable to support any studies which suggest that NSILA can be converted to immunoreactive insulin. In none of the fractions which we studied was any significant amount of IRI demonstrated by immunoassay. In one acid ethanol extracted fraction insulin-antibody binding was depressed using the double antibody method but not when assayed by cellulose chromatography or ethanol precipitation. It was concluded that the extract interfered with the second antibody reaction. Since the suppression of column effluent serum ILA by guinea pig antisera in the biossay system could not be substantiated by immunoassay, it was concluded that this was an artifact.

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