# EVIDENCE FOR THE EXISTENCE IN HUMAN SERUM OF LARGE MOLECULAR WEIGHT NONSUPPRESSIBLE INSULIN-LIKE ACTIVITY (NSILA) DIFFERENT FROM THE SMALL MOLECULAR WEIGHT FORMS

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## 1. Introduction

In several biological in vitro systems such as the rat diaphragm [1], rat epididymal adipose tissue [2] or isolated fat cells [3,4] human serum exerts insulin-like effects which cannot be 'neutralized' by the addition of insulin antibodies. This activity has been designated as nonsuppressible insulin-like activity (NSILA) [2]. Two fractions of NSILA had been obtained earlier by acid/ethanol extraction of acetone powder prepared from human serum: one of small molecular weight, soluble in acid/ethanol, which was called NSILA-S [5]; and one of large molecular weight, precipitated by acid/ethanol, and termed NSILA-P in [5]. NSILA-S has been further purified. It has been shown to contain 2 insulin-like polypeptides (termed insulin-like growth factors, IGF I and II) whose chemical [6–8] and biological properties [9] have been defined. The 2 polypeptides occur in human serum in tight association with a specific carrier protein [10] from which they dissociate under acidic conditions [11,12]. Doubts have, however, been raised about the existence of NSILA-P. Chromatography of human serum on Sephadex G-75 in 1 M acetic acid, did not reveal measurable amounts of large mol. wt NSILA. They recovered all the insulin-like activity in a small mol. wt fraction (mol. wt <10 000) [11]. Recently, Poffenbarger, using Dowex-50 ion exchange chromatography as the first purification step, isolated a large mol. wt insulin-like protein (NSILP) which, like NSILA-P, could no longer be dissociated into small mol. wt NSILA by acid treatment [13].

In this work we tried to reassess the problem with the methods applied in our earlier studies [11,12]. Avoiding lyophilization of the large mol. wt serum fractions in acetic acid we were able to demonstrate that, besides small mol. wt NSILA bound to carrier protein, human serum contains considerable amounts of large mol. wt NSILA which cannot be converted to the small mol. wt form under acidic conditions and which does not cross-react with $^{125}$I-labelled NSILA-S on the NSILA-carrier protein.

## 2. Materials and methods

### 2.1. *Serum chromatography*

A. Four 20 ml samples of a normal human serum pool were chromatographed on a Sephadex G-200 column (bed vol. 1000 ml, diam. 4 cm) in 0.1 M phosphate buffer, pH 7.4. Fractions were pooled between 30% and 40% and then every 5% of the bed volume, concentrated by ultrafiltration to 4–6 ml and dialyzed against Krebs-Ringer bicarbonate buffer. In each of these serum fractions total NSILA was determined in the presence of 2 μl guinea pig anti-insulin serum (insulin-neutralizing capacity 2 mU) in the fat pad assay [2] and in a fat cell (FC) assay which measures the stimulation of lipid synthesis from [U-$^{14}$C]glucose as in [14–16]. Different dilutions of crystalline porcine insulin served as reference standards.

B. Samples, 1.5 ml, of each of the neutral concentrated serum fractions obtained in A, were chromatographed on Sephadex G-50 (bed vol. 100 ml,

diam. 1 cm) in 1 M acetic acid. This procedure has been shown to result in dissociation of small mol. wt NSILA from its carrier protein [11,12,17–19]. Fractions eluting between 30% and 50% bed vol. were pooled (30–50% pools), concentrated by ultrafiltration, dialyzed against Krebs-Ringer bicarbonate buffer, and NSILA was measured in the fat pad and FC assays (see A). The fractions eluting between 50–55%, 55–60% and 60–85% bed vol. were pooled, lyophilized, dissolved in 2 ml 0.1 M $(NH_4)_2CO_3$, and NSILA was determined by a competitive protein binding assay using $^{125}$I-labelled NSILA-S as a tracer and different dilutions of unlabelled NSILA-S (3.8 mU/mg) for standardization as detailed in [12]. Aliquots, 0.7 ml, of the 3 pools were then recombined in 1 pool (50–85% pools), relyophilized and dissolved in Krebs-Ringer bicarbonate buffer containing 0.2% human serum albumin (HSA). NSILA was then determined biologically in the fat pad and FC assays.

### 2.2. *Determination of NSILA, of $^{125}$I-labelled NSILA-S binding activity and presentation of results*

All NSILA determinations were done in duplicate and, when the NSILA concentration allowed it, at 2 different dilutions. In A, the results were expressed as total μU NSILA (in terms of insulin equivalents in the fat pad or the FC assay) per serum fraction. In B, NSILA was calculated as μU contained in the 30–50% or 50–85% pools of the whole respective serum fraction from which they were obtained. Again, μU measured in the fat pad and FC assays refer to insulin equivalents. The competitive protein binding assay can only be applied to the determination of small mol. wt NSILA in the absence of contaminating binding protein [12]. Therefore, only 50–85% pools could be assayed. μU determined in the protein binding assay refer to NSILA-S as the standard. There is a good correlation between the fat pad and the protein binding assay, although 2-fold higher than in the fat pad assay [12], (table 1). For unknown reasons, the results of the FC assay are also 2-fold higher than those of the fat pad assay and usually agree with those obtained in the protein binding assay.

Specific $^{125}$I-labelled NSILA-S binding was determined in 2% bed vol. fractions after neutral Sephadex G-200 chromatography of serum (A) and in aliquots of 30–50% pools (B) (after dialysis against 0.1 M phosphate buffer, pH 7.0) obtained after removal of small mol. wt NSILA by acidic chromatography of the neutral fractions. The method for the determination of $^{125}$I-labelled NSILA-S binding activity has been detailed in [10,19]. Total binding (in the absence of added unlabelled NSILA-S) and nonspecific binding (in the presence of excess unlabelled NSILA-S: 250 μU/ml) were measured in duplicate at different dilutions of the samples in order to find out the range where specific binding (difference between total and nonspecific binding) became linearly related to the dilution. Specific binding was then expressed as 'relative specific $^{125}$I-labelled NSILA-S binding activity' (cpm bound/fraction or pool).

The iodination procedure for NSILA-S as well as the characteristics of the labelled preparation have been described [12,20].

## 3. Results and discussion

The results of this study are presented in fig.1A and 1B and in table 1. Unextracted normal whole serum contains ~200 μU/ml of NSILA as determined in the fat pad assay [11,21]. The serum pool used in this study contained 195 μU/ml. After passage over Sephadex G-200 at neutral pH the recovery was 276%, a still unexplained observation made also in [21]. The peak of NSILA eluted between 50% and 60% bed vol. (mol. wt 90 000–160 000) as in [21]. This was surprising, since we should have expected 2 NSILA peaks coinciding with the 2 major $^{125}$I-labelled NSILA-S binding peaks at $K_{av}$ 0.2 and 0.5 (47% and 66% bed vol.). When the NSILA peak fractions between 50–55% and 55–60% bed vol. were chromatographed on Sephadex G-50 in 1 M acetic acid the total recovery of NSILA (30–50% plus 50–85% pools, fig.1B) was 77% (41% in the FC assay) (table 1). However, only 12% (563 μU; FC assay: 1560 μU = 15%) and 7% (286 μU; FC assay: 234 μU = 3%), respectively appear as small mol. wt NSILA in the 50–85% pools, whereas the rest (3042 μU and 2627 μU; FC assay: 2824 μU and 2413 μU) elutes in the 30–50% pools (table 1). The amounts of NSILA measured in the protein binding assay in the 50–85% pools (1093 μU and 288 μU) agree with those determined in the FC assay (table 1).

Table 1
NSILA in serum fractions obtained by neutral Sephadex G-200 chromatography (A) and in the 30–50% and 50–85% pools obtained by chromatography on Sephadex G-50 in 1 M acetic acid (B) of the serum fractions from A

| | A | | B | | | | |
|---|---|---|---|---|---|---|---|
| | NSILA in serum fractions obtained by neutral Sephadex G-200 chromatography (μU/fraction) | | NSILA in 30–50% and 50–85% pools obtained by chromatography of serum fractions from A on Sephadex G-50 in 1 M acetic acid (μU/pool) | | | | |
| Fractions from % bed vol. | Fat pad assay | Fat cell assay | Fat pad assay | | Fat cell assay | | Protein binding assay 50–85% |
| | | | 30–50% | 50–85% | 30–50% | 50–85% | |
| 30–40 | 306 ± 21 | 625 ± 156 | <100 | <100 | <100 | <100 | 31 ± 3 |
| 40–45 | 199 ± 60 | 339 ± 74 | 127 ± 14 | <100 | 157 ± 47 | <100 | 146 ± 52 |
| 45–50 | 936 ± 4 | 1574 ± 500 | 270 ± 22 | 567 ± 95 | 368 ± 143 | 1024 ± 192 | 1071 ± 139 |
| 50–55 | 4674 ± 464 | 10 677 ± 3230 | 3042 ± 485 | 563 ± 146 | 2824 ± 402 | 1560 ± 396 | 1093 ± 167 |
| 55–60 | 3848 ± 734 | 6847 ± 1602 | 2627 ± 141 | 286 ± 124 | 2413 ± 407 | 234 ± 39 | 288 ± 48 |
| 60–65 | 521 ± 30 | 1318 ± 178 | 394 ± 100 | 241 ± 14 | 334 ± 39 | 351 ± 53 | 321 ± 44 |
| 65–70 | 238 ± 15 | 305 ± 80 | <100 | <100 | <100 | 172 ± 122 | 138 ± 82 |
| 70–75 | 52 ± 23 | <100 | <100 | <100 | <100 | <100 | 33 ± 20 |
| Total | 10 774 | 21 685 | 6460 | 1657 | 6069 | 3441 | 3121 |
| | | | 8117 | | 9537 | | |

The procedures are the same as described in fig.1A,B. In addition to the results of the fat pad assay, the results of the fat cell assay and of the protein binding assay are given. Results represent the mean of 4 different experiments with SEM values

Fig.1A

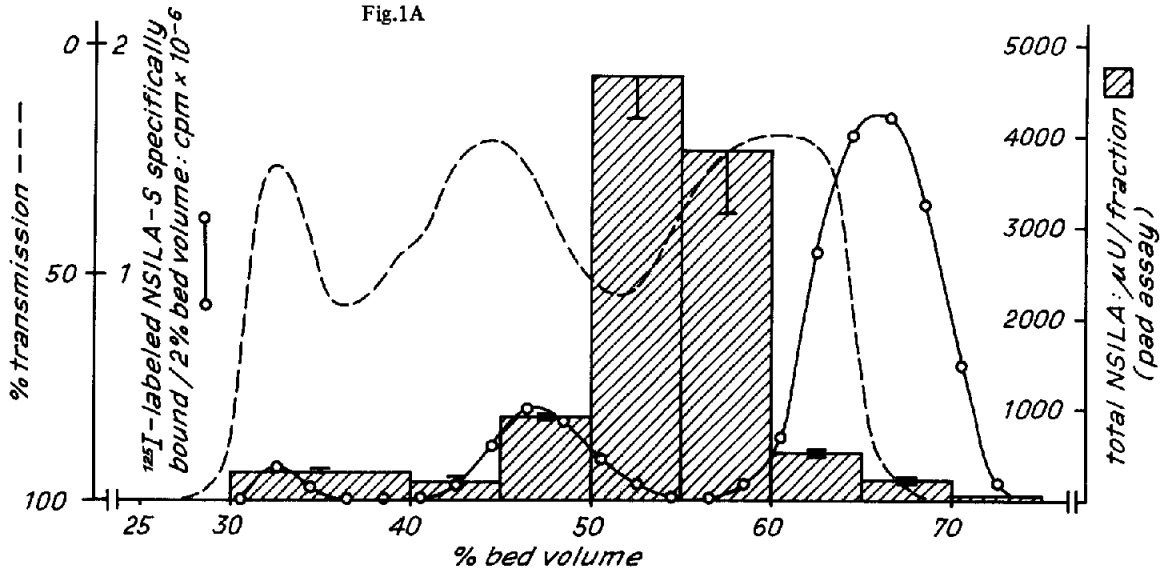


Fig.1A. Total NSILA and relative $^{125}$I-labelled NSILA-S binding activities in fractions of human serum after chromatography on Sephadex G-200 in 0.1 M phosphate buffer, pH 7.4. Normal serum, 20 ml, was chromatographed on Sephadex G-200 at neutral pH. Specific $^{125}$I-labelled NSILA-S binding activity (o———o) was measured in 2% bed vol. fractions. NSILA (hatched columns) was determined by the rat fat pad assay in 5% bed vol. fractions. All determinations were carried out in duplicate and, for NSILA, at 2 different dilutions. 4 different experiments were carried out with the same pool of normal human serum. Bars give the SEM of these 4 experiments.

In contrast, when the binding peak fraction between 45% and 50% bed vol. (after neutral chromatography on Sephadex G-200) is chromatographed in 1 M acetic acid, 60% (567 μU) of its NSILA is dissociated, apparently representing carrier-bound small mol. wt form, whereas only 29% (270 μU) elute in the 30–50% pool (fig.1B and table 1). The results of the FC assay give a similar picture (1024 μU = 65% in the 50–85% pool, 268 μU = 23% in the 30–50% pool) and agree with those of the protein binding assay (1071 μU in the 50–85% pool).

More NSILA binding sites should become available on the binding protein after dissociation of small mol. wt NSILA. This is indeed the case: the specific relative $^{125}$I-labelled NSILA-S binding activity of the binding fractions between 40% and 55% bed vol. increases ~20-fold, that of the binding fractions between 55% and 75% bed vol., which contain less dissociable NSILA, only ~2-fold. Since the 2 binding peaks in A overlap with the NSILA peak and since rechromatography of the 30–50% pools of the NSILA peak in 1 M acetic acid does not result in further measurable dissociation of small mol. wt NSILA, we assume that the small mol. wt NSILA dissociated from the NSILA peak fractions (50–60% bed vol. in A) also represents NSILA originally associated with binding protein. By the same token, large mol. wt NSILA recovered in the 2 binding peaks probably belongs to the NSILA peak. Altogether, this means that most of the dissociable small mol. wt NSILA (70–80%) is carried by the binding protein fraction with $K_{av}$ 0.2 and only 20–30% by the one with $K_{av}$ 0.5 (fig.1A).

These findings together with those in [13] strongly favour the earlier concept [5] according to which at least 2 forms of NSILA are present in human serum:

1. Carrier-bound NSILA, which dissociates as small mol. wt NSILA under acidic conditions (10,19)

2. Large mol. wt NSILA, which is not interconvertible to the small mol. wt form by acid treatment.

However, whereas we detected ~16% of the total

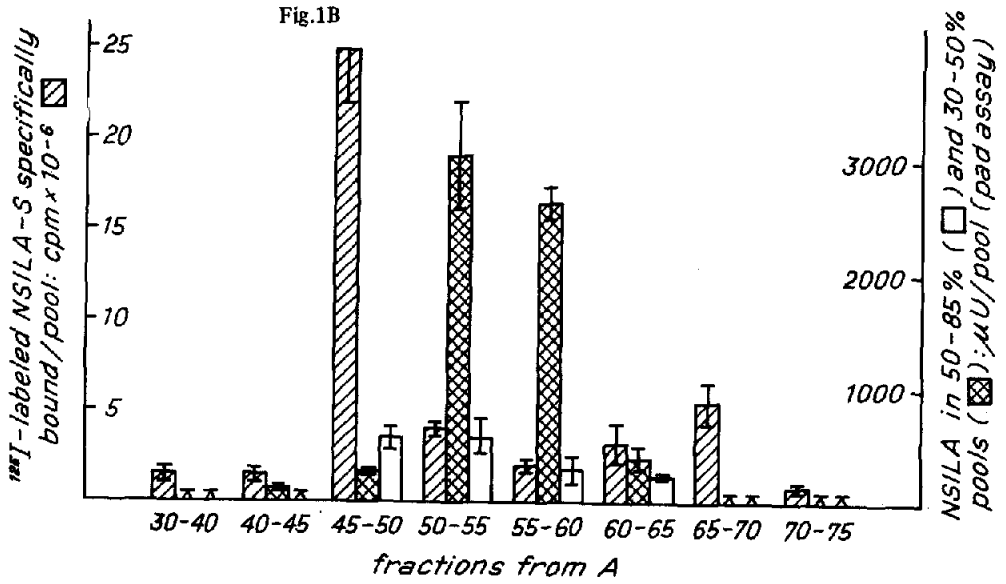


Fig.1B. NSILA in 50–85% (small mol. wt NSILA) and in 30–50% pools (large mol. wt NSILA), and relative $^{125}$I-labelled NSILA-S binding activities in 30–50% pools, obtained by chromatography on Sephadex G-50 in 1 M acetic acid of the serum fractions from experiment A. Ultrafiltered 5% bed vol. fractions from A were passed over Sephadex G-50 in 1 M acetic acid. Fractions were pooled between 30–50% and 50–85% bed vol. as in section 2. Specific $^{125}$I-labelled NSILA-S binding activity (hatched columns) and NSILA (rat fat pad assay; cross-hatched columns) were determined in the 30–50% pools after ultrafiltration on dialysis (see text). Small mol. wt NSILA (open columns) was measured in the lyophilized 50–85% pools by the fat pad assay (see also table 1). The height of the columns gives the mean of 4 different experiments, each started from step IA. Bars give the SEM of these 4 experiments. Triangles indicate NSILA values below the limit of sensitivity of the assay.

NSILA, recovered after fractionation of serum by neutral Sephadex chromatography, to be bound to carrier protein and to be dissociable as small mol. wt NSILA, it could not be detected in [13]. Recent investigations (Kaufmann, J. Z. and E. R. F., in preparation) indicate that this seems to be due to the different methodological approach. The explanation why we did not find large mol. wt NSILA during recent years lies in the fact that all serum fractions obtained by acidic Sephadex chromatography were lyophilized in 1 M acetic acid. This causes a nearly total loss of large mol. wt NSILA. Lyophilization at neutral pH, however, does not result in losses of its biological activity.

We do not yet know whether the large mol. wt NSILA fraction described here is identical to NSILA-P [5] or to NSILP [13], or whether it is a precursor of NSILA-S. So far, we have not yet made an attempt to further characterize it physico-chemically or to test it for other biological effects. Preliminary studies have shown that the partially purified large mol. wt NSILA fraction, devoid of $^{125}$I-labelled NSILA-S binding activity, does not cross-react with $^{125}$I-labelled NSILA-S in the protein binding assay nor with $^{125}$I-labelled IGF I in an IGF I-radioimmunoassay (unpublished observation).

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