Preferential association of the insulin-like growth factors I and II with metabolically inactive and active carrier-bound complexes in serum

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Ion-exchange chromatography of serum on DEAE-Sephadex A-50 using a stepwise NaCl gradient showed that complexes enriched with insulin-like growth factors I and II (IGF-I and IGF-II) could be preferentially eluted. A fraction eluted with 0.075 M-NaCl preferentially contained immunoreactive IGF-I with peak levels appearing in fractions of M_r approx. 110000. The IGF-I–binding protein complex itself had low bioactivity as measured in a non-suppressible insulin-like (NSILA) bioassay. On conversion to free IGF-I by gel-permeation chromatography on Sephadex G-75 in 1% formic acid, however, the IGF-I did express its intrinsic NSILA bioactivity. In contrast, an IGF-II-enriched complex was eluted from the DEAE-Sephadex with 0.15 M-NaCl. Practically all of the recovered NSILA of the original serum was present in this fraction, in the M_r range 70000–300000 with a peak of 150000. Chromatography on Sephadex G-75 in 1% formic acid separated this high-M_r NSILA into low-M_r (< 15000) IGF-II and high-M_r acid-stable NSILA-P. The high-M_r IGF-II complex bound to concanavalin A–Sepharose, suggesting that it was a glycoprotein. The results confirm previous reports that a large portion of the NSILA of whole serum can be accounted for by a biologically active acid-dissociable complex. These data show for the first time that this active complex consists of an IGF-II-preferring binding protein. In direct contrast, the IGF-I-preferring complex does not express NSILA bioactivity until the IGF-I is liberated through acidification. The presence of a metabolically active IGF-II complex in serum raises questions as to its possible biological role in the adult.

INTRODUCTION

Non-suppressible insulin-like activity (NSILA) of whole serum has, in the past, been ascribed to a high-M_r acid-stable form, NSILA-P or NSILP (Jacob *et al.*, 1968; Poffenbarger, 1975). A second low-M_r form, NSILA-S, is also present, but is generally considered not to contribute to the total NSILA of whole serum because it is normally bound to carrier proteins (Zapf *et al.*, 1975; Hintz & Liu, 1977). NSILA-S, although active in the free form, has been shown to be inhibitable by addition of crude binding protein fractions (Meuli *et al.*, 1978; Zapf *et al.*, 1979). NSILA-S includes the insulin-like growth factors IGF-I and IGF-II (Rinderknecht & Humbel, 1976) and probably a two-chain acidic form (ILA pI 4.8) (Cockerill *et al.*, 1980; Herington & Kuffer, 1984). In somatomedin terminology, somatomedin C is identical with IGF-I (Klapper *et al.*, 1983) and somatomedin A is a deamidated form of IGF-I (Enberg *et al.*, 1984).

The major carrier protein in normal serum appears to be of $M_r \sim 150000$ (Zapf *et al.*, 1978) and comprises at least two subunits; a binding protein and an acid-labile component (Furlanetto, 1980; Hintz & Liu, 1980). More recent studies (Wilkins & D'Ercole, 1985) have suggested this carrier protein is a hexamer of M_r 24000–28000 IGF-binding protein subunits. Plasma also contains an acid-stable somatomedin binding protein with a M_r of 35000–50000 (Hintz *et al.*, 1981). It appears that experimental conditions, especially the pH, have a strong influence on the M_r of the binding protein species detected, as Morris & Schalch (1982) found M_r-30000 and M_r-46000 species after alkaline exposure. It is believed that the binding proteins may modulate the biological effects of the IGF/somatomedin group of substances (Meuli *et al.*, 1978; Zapf *et al.*, 1979). However, a precise role for IGF-binding proteins has not yet been established (for reviews see Herington *et al.*, 1983; Smith, 1984).

We have recently suggested (Cornell & Herington, 1983) that there is a significant contribution to the NSILA of whole serum by a high-M_r form which consists of low-M_r NSILA bound to a carrier protein. This complex was dissociable by treatment with 1% formic acid (pH 2.4). Evidence was obtained by quantitative gel permeation chromatography of Cohn fraction IV-I that serum NSILA recovered under neutral conditions was all high-M_r and could not be accounted for by the acid-stable high-M_r (NSILA-P) form recovered under acid conditions. The difference in activity (about 2-fold) appeared to be due to an active carrier-bound form of NSILA-S. Subsequent studies (Cornell *et al.*, 1985) with serum confirmed these findings and indicated that the bioactive NSILA–carrier protein complex was found in gel permeation chromatographic fractions of M_r 35000–200000 and was also bound by Con-A–Sepharose.

We have now further characterized the high-M_r

Abbreviations used: IGF, insulin-like growth factor; NSILA, non-suppressible insulin-like activity; Con-A, concanavalin A.
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bioactive NSILA-binding complex and in particular have determined whether it is associated with IGF-I and/or IGF-II. In addition, its relationship to the other forms of NSILA present, i.e. the acid-stable form (NSILA-P) and the high-M_r inactive form (which releases NSILA-S after treatment with acid) have been explored.

EXPERIMENTAL

Ion-exchange chromatography

Pooled serum (22 ml) from approx. 250 normal healthy adult blood bank donors was diluted in an equal volume of 0.05 M-Tris/HCl buffer, pH 8.1, and applied to a column (2.6 cm × 36 cm) of DEAE-Sephadex A-50 (Pharmacia, Uppsala, Sweden) equilibrated in the same buffer. The chromatogram was developed at a flow rate of 50 ml/h, using a stepwise gradient of 0.075M-, 0.15 M-, 0.25 M- and 0.6 M-NaCl as shown in Fig. 1. Fractionation was monitored at 280 nm. Material in the main peaks, together with material between the peaks, was dialysed over 60 h with four changes of distilled water at 6 °C and then lyophilized.

Neutral pH gel-permeation chromatography

Selected fractions (B and C) from ion-exchange chromatography (30–60 mg dry wt.) were chromatographed on a column (1.6 cm × 80 cm) of Sephacryl S-300 fine (Pharmacia) equilibrated with 0.05 M-Tris/HCl (pH 7) containing 0.1 M-NaCl. The column was calibrated using NaCl, glucagon, human growth hormone, bovine serum albumin, rabbit muscle aldolase and Dextran Blue. After dialysis and lyophilization, fractions were assayed for IGF-I and -II and NSILA as described below.

Acidic pH gel-permeation chromatography

Fractions B and C (30–50 mg dry wt.) were dissolved in 1% (v/v) aqueous formic acid (1 ml) and after 2 h, an aliquot was taken for assay and the remainder was chromatographed on a column (1.2 cm × 44 cm) of Sephadex G-75 (Pharmacia) equilibrated in 1% (v/v) aqueous formic acid (pH 2.4). Chromatography was carried out at a flow rate of 6 ml/h. Fractions of $K_{av.}$ 0–0.4 and $K_{av.}$ 0.4–1.0 were each pooled and dialysed, together with the aliquot of applied sample, using Spectropor 3 tubing (Spectrum Medical Industries Inc.). The lyophilized fractions were assayed for NSILA as described below.

Affinity chromatography on Con-A–Sepharose

Fraction C (50 mg dry wt.) was chromatographed on Con-A–Sepharose (Pharmacia) as described previously (Cornell & Herington, 1983). Briefly, the unbound fraction was obtained in loading buffer (0.05 M-Tris/HCl, pH 7.5, containing 0.1 M-NaCl and also 1 mM-$CaCl_2$, 1 mM-$MnCl_2$ and 1 mM-$MgCl_2$). The bound fraction was obtained by application of the same buffer containing a mixture of α-methyl glucoside and α-methyl mannoside (each 0.05 M) in the same buffer and the strongly bound fraction by application of the glycosides (each 0.075 M) in the same buffer containing additional NaCl (to 0.35 M) and acetic acid to pH 5.5. These fractions were assayed for IGF-I and -II as described below.

Assays

Radioimmunoassays for IGF-I and -II in fractions from ion-exchange chromatography of serum were carried out according to the methods of Hall *et al.* (1979) and Enberg & Hall (1984) using IGFs prepared from human serum (Enberg *et al.*, 1984) as standards and iodinated tracer. The fractions were also subjected to a radio-receptor assay utilizing human placental membranes and ^{125}I-labelled IGF-I (Takano *et al.*, 1976). The standard used was a normal reference serum with an activity of 1 somatomedin unit/ml.

NSILA in the fractions was determined in a standard

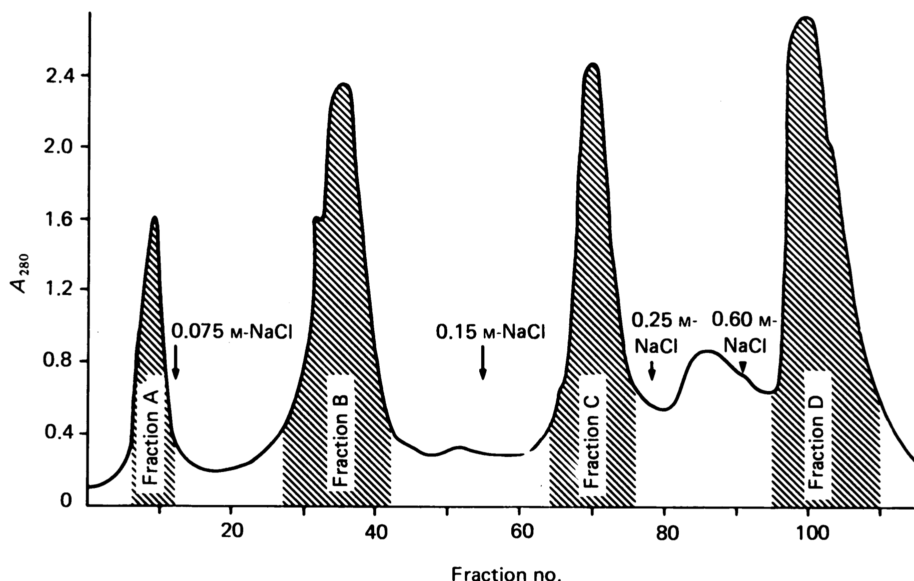

Fig. 1. Chromatogram of normal human serum on DEAE-Sephadex A-50

Starting buffer was 0.05 M-Tris/HCl, pH 8.1. Other buffers contained, in addition, 0.075 M-, 0.15 M-, 0.25 M- and 0.60 M-NaCl applied as shown by arrows. Column dimensions, 36 cm × 2.6 cm; flow rate, 50 ml/h; fraction volume, 12 ml; sample size, 22 ml of serum.

Table 1. Recovery of immunoreactive IGF-I and -II in major protein fractions from chromatography of serum on DEAE-Sephadex A-50 in comparison with their NSILA.

Mean percentages of total activity recovered (±S.E.M.) are shown for three separate chromatographic runs.

Fraction	Recovered (%)		
	IGF-I	IGF-II	NSILA
A (eluted in starting buffer)	3±1	1±1	0±0
B (eluted in 0.075 M-NaCl)	67±2	21±7	5±3
C (eluted in 0.15 M-NaCl)	13±4	65±9	89±4
D (eluted in 0.25 M-/0.60 M-NaCl)	6±3	1±1	6±4

NSILA-S bioassay by monitoring the incorporation of [U-^{14}C]glucose into lipid by isolated rat adipocytes in the presence of excess anti-insulin serum (Franklin *et al.*, 1976). Porcine insulin (24 units/mg) was used as standard (assayed in the absence of anti-insulin serum) and all bioassay results are expressed as m-units of insulin equivalents.

RESULTS

Fig. 1 shows the elution profile for the chromatography of serum on DEAE-Sephadex A-50. Major peaks were observed corresponding to material eluted with starting buffer (fraction A), 0.075 M-NaCl (fraction B), 0.15 M-NaCl (fraction C) and 0.25 M-NaCl or higher (fraction D).

Table 1 shows the results of the bioassays for NSILA and immunoassays for IGF-I and -II on fractions B and C from DEAE-Sephadex A-50 chromatography. They clearly show a strong association between the NSILA bioactivity and IGF-II content (fraction C). On the other hand, high levels of IGF-I were observed in fraction B, but the NSILA of this fraction represented only 5% of the total recovered. Fraction D contained the remainder (6%) of the total NSILA recovered. Bioactivity and/or immunoactivity of the other fractions and of material between the peaks indicated generally low levels of activity and the data are not shown for reasons of simplicity. Radioreceptor assay of the fractions showed that major amounts of activity were present in fractions B and C (16 and 12 somatomedin units recovered respectively). The results of immunoassays for IGF-I and -II in different M_r ranges following neutral gel permeation chromatography of fraction B from ion-exchange chromatography are shown in Table 2. As expected, IGF-I was the major component (83%) of the total IGFs recovered. IGF-I was observed mainly in fractions of M_r 25000–250000 with 69% of that recovered being in fractions 4 and 5, covering the M_r range 60000–150000. The small amount of IGF-II detected was found almost entirely in fractions 5 and 6, particularly the latter (M_r 25000–60000).

Table 3 shows the results of immunoassays and NSILA bioassays carried out on different M_r ranges following neutral gel permeation chromatography of fraction C. The NSILA is highest (86% of total recovered) in fraction 4 (M_r 120000–170000). Interestingly, IGF-II was the major form present in fraction 4 (76% of the total IGFs in this fraction) and it contains the major part (57%) of the IGF-II present in fraction C. The next highest amount of IGF-II (31%) was in fraction 5, but this fraction had minimal NSILA. IGF-I was observed mainly in fractions of M_r 70000–300000 (fractions 3–5) but was present to the extent of only 24% of the total IGFs in the fraction containing the highest NSILA (fraction 4).

Acidic gel-permeation chromatography of fraction B (the IGF-I-enriched fraction) (Table 4) showed that appreciable NSILA was recovered in the low-M_r (< 15000) fraction, while no activity was recovered in the high-M_r fraction (> 15000). The activity of the samples applied was very small, being undetectable in one experiment.

Table 5 shows that when fraction C (the IGF-II-enriched fraction) was subjected to acidic-gel permeation chromatography, 27% of the total recovered NSILA, on average, was obtained in the high-M_r fraction. Mean

Table 2. Immunoreactive IGF-I and IGF-II in fractions of different M_r derived from Sephacryl S-300 chromatography of fraction B

The starting material was 40 mg dry wt. of fraction B (the 0.075 M-NaCl elutable material from DEAE-Sephadex A-50 chromatography of serum). Values in parentheses are the amounts of IGF-I or IGF-II in each fraction, expressed as percentages of the total of each factor recovered.

Fraction no.	10^{-3} × M_r range	IGF-I (ng)	IGF-II (ng)
1	> 400	3 (1)	3 (6)
2	250–400	3 (1)	0 (0)
3	150–250	36 (14)	0 (0)
4	100–150	99 (38)	0 (0)
5	60–100	80 (31)	16 (31)
6	25–60	38 (15)	33 (63)

Table 3. Immunoreactive IGF-I and IGF-II in comparison with NSILA in fractions of different M_r derived from Sephacryl S-300 chromatography of fraction C

The starting material was 60 mg dry wt. of fraction C (the 0.15 M-NaCl elutable material from DEAE Sephadex A-50 chromatography of serum). Values in parentheses are the amounts of IGF-I, IGF-II or NSILA in each fraction, expressed as percentages of the total of each factor recovered.

Fraction no.	10^{-3} × M_r range	IGF-I (ng)	IGF-II (ng)	NSILA (m-unit of insulin equivalents)
1	> 500	18 (5)	5 (1)	0 (0)
2	300–500	28 (7)	8 (1)	0 (0)
3	170–300	90 (24)	35 (5)	0.03 (5)
4	120–170	120 (31)	392 (57)	0.48 (86)
5	70–120	114 (30)	212 (31)	0.05 (9)
6	< 70	12 (3)	36 (5)	0 (0)

Table 4. Recovery of NSILA in fractions obtained by chromatography of fraction B on Sephadex G-75 in 1% formic acid

The starting material was 40 mg dry wt. of fraction B. Values in parentheses are percentages of recovered activity.

Experiment	Activity applied (m-units of insulin equivalents)	Activity (m-unit of insulin equivalents) in fraction of $K_{av.}$:	
		0–0.4	0.4–1.0
1	0.012	0 (0)	0.235 (100)
2	Not detectable	0 (0)	0.384 (100)

total recovery was 98% of the activity applied to the column. This result indicates that fraction C contains both an active IGF-II–carrier complex and acid-stable NSILA-P.

The results of Con-A–Sepharose affinity chromatography of fraction C are presented in Table 6. They clearly indicate a high proportion (88%) of IGF-I in the unbound fraction and, in direct contrast, a high proportion (95%) of IGF-II in the bound fractions. There is naturally some batch-to-batch variation in the IGF-I/IGF-II ratio of the fraction C used. In one experiment the bound fractions contained 19% IGF-I of the total IGFs and in this case, the IGF-I recovered in the parent fraction C used was 20%, whereas for the tabulated results, the IGF-I recovery in the parent fraction was only 5%.

To determine the M_r range of activity in the fractions which bound to Con-A–Sepharose, these fractions were subjected to neutral pH Sephacryl chromatography. Peak levels of both IGF-I and IGF-II were found in the fraction of M_r 70000–120000 (35% and 50% of the recovered IGF-I and IGF-II respectively). Levels of IGF-II were also high (32% of the recovered IGF-II) in the fraction of $M_r < 70000$. The recovery of IGF-II in these fractions was over double that of IGF-I (results not shown).

DISCUSSION

The chromatographic separation of the IGFs and NSILA can be summarized in Fig. 2. A good separation of IGF-I, IGF-II and NSILA-P can be accomplished in two steps as shown.

The results of assays on fractions from the DEAE-Sephadex A-50 chromatography show a significant separation of the carrier-bound forms of IGF-I and IGF-II immunoreactivity, the latter being associated with NSILA bioactivity. This separation was accomplished by use of different concentrations (0.075 M and 0.15 M) of NaCl. Furlanetto (1980) eluted most of the somatomedin C (IGF-I) activity of a 35–55% $(NH_4)_2SO_4$ precipitate of serum by application of a Tris buffer containing 0.15 M-NaCl. Presumably, his fraction (Peak 2) also contained the IGF-II activity.

In the present work, the use of 0.075M-NaCl allowed the separation of an IGF-I-enriched fraction (fraction B) responsible for only 5% on average, of the recovered high-M_r NSILA. This fraction had peak levels of IGF-I immunoreactivity at M_r 110000 (range 25000–250000). These results are in agreement with those of Wilkins & D'Ercole (1985) who found several ^{125}I-somatomedin C–binding protein complexes in serum with relative M_r values of 160000, 135000, 110000, 80000, 50000, 35000–43000 and 24000–28000. All except the M_r 35000–43000 complex appeared to be growth-hormone-dependent and were bound by Con-A–Sepharose. They speculated that the somatomedin C binding protein was an oligomer composed of several identical subunits, each with M_r 24000–28000.

A major finding of the present study was that appreciable amounts of low-M_r NSILA (NSILA-S) were obtained from fraction B after acidic gel-permeation chromatography, corresponding to about 0.08 m-unit of insulin equivalents/ml of serum. This finding is consistent with that of Chatelain *et al.* (1983) who obtained increases in immunoreactive somatomedin C levels after incubation of serum in the presence of acid.

Thus, it is likely that most of the IGF-I of serum is bound to a carrier protein of $M_r \sim 100000$ and that this complex represents an inactive form of NSILA in serum. The NSILA activity in such a complex might be released by a protease (Chatelain *et al.*, 1983) under physiological conditions when required by the organism. The IGF-I-rich fraction B, therefore, is worthy of additional study, especially to purify the material further and to determine physiological conditions for release of its NSILA bioactivity. The importance of studies on IGF-I and its carrier-bound forms is emphasized by its clear dependence on growth hormone (Zapf *et al.*, 1981), and its ability to induce skeletal growth in hypophysectomized rats

Table 5. Recovery of NSILA in fractions obtained by chromatography of fraction C on Sephadex G-75 in 1% formic acid

The starting material was 30–50 mg dry wt. of fraction C. Total recovery of NSILA in fractions is also shown.

Experiment	Activity applied (m-unit of insulin equivalents)	NSILA recovered (% of total recovered) in fraction of $K_{av.}$		Total recovery (%)
		0–0.4	0.4–1.0	
1	0.654 (50 mg)	18	82	107
2	0.337 (35 mg)	32	68	95
3	0.267 (30 mg)	32	68	91
Means ± S.E.M.		27±5	73±5	98±5

Table 6. Immunoreactive IGF-I and IGF-II in fractions obtained by affinity chromatography of fraction C on Con-A–Sepharose

The starting material was 50 mg dry wt. of fraction C. Values in parentheses are percentages of IGF-I and -II of the total recovered in each case.

Fraction	IGF-I (ng)	IGF-II (ng)
Unbound	144 (88)	24 (5)
Bound	0 (0)	390 (77)
Strongly bound	20 (12)	92 (18)

(Schoenle *et al.*, 1982). IGF-II on the other hand has little effect on skeletal growth in these animals (Schoenle *et al.*, 1985).

In the light of previous experiments (Cornell & Herington, 1983; Cornell *et al.*, 1985), it is apparent that the NSILA of whole serum is due partly to an acid-dissociable complex of IGF-II bound to a carrier glycoprotein and partly to the acid-stable NSILA-P. Both these forms of NSILA were co-eluted from DEAE-Sephadex in fraction C by the application of buffer containing 0.15 M-NaCl and gave rise to just one peak of NSILA.

One peak of NSILA was also obtained in previous experiments where serum was directly chromatographed on Sephacryl S-300 (Cornell *et al.*, 1985), indicating that the molecular size of the two forms of NSILA in serum overlap; indeed, activity was observed over a wide range of M_r (50000–400000) with a peak at 200000. In the present work, chromatography on Sephacryl S-300 columns indicated that the IGF-II was present mainly in fractions of M_r 70000–120000 and 120000–170000. Peak NSILA was observed in the latter fraction at $M_r \sim 150000$. These results are almost identical with those of Burgi *et al.* (1966), who found that NSILA eluted exclusively with proteins of M_r 70000–150000 and that NSILA could not be detected with the fat pad assay in the low-M_r region. They are, however, a little different from our previous results obtained from direct chromatography of serum on Sephacryl S-300, where activity was obtained over a wider range with a peak of $M_r \sim 200000$.

Acidic gel-permeation chromatography confirmed the presence of high-M_r acid-stable NSILA in fraction C, being on average 27% of the total NSILA recovered. In three experiments, mean recovery of the applied activity (98%) was quite consistent with that of previous experiments (Cornell *et al.*, 1985) where there was a tendency for the yields of activity to be greater than the applied activity (mean of five experiments was 112%). In these latter experiments, the yield of the acid-stable NSILA was also higher, further supporting the suggestion that very-high-M_r forms of the acid-stable NSILA have been removed in producing fraction C. The remaining 73% of NSILA recovered from fraction C in the low-M_r range was presumably due to IGF-II released from the carrier glycoprotein.

The affinity chromatography experiments confirmed the results of previous work (Cornell *et al.*, 1985), which showed that the active acid-dissociable complex was bound by Con-A–Sepharose. The material in fraction C which bound to Con-A contained almost all (95%) of the IGF-II present in the fraction. By contrast, the IGF-I was found chiefly in the unbound fraction (88% of the IGF-I in fraction C). As expected, the amount of IGF-I recovered from this fraction compared with IGF-II was relatively low, being 24% of the total IGFs. It is not known whether the IGF-I present in the unbound fraction from Con-A–Sepharose chromatography represents a further portion of the inactive complex found in fraction B. However, previous experiments (Cornell &

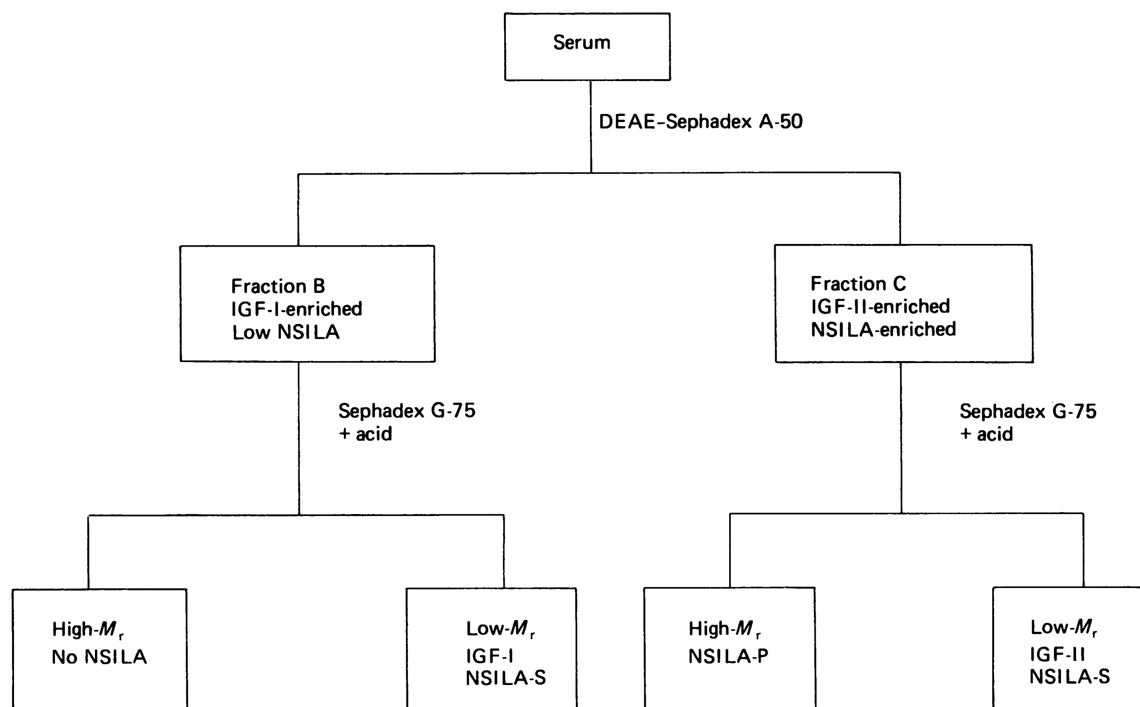

Fig. 2. Chromatographic separation of IGFs/NSILA in serum.

Herington, 1983) indicated inactive acid-dissociable complex in unbound fractions from Con-A–Sepharose.

When the first bound fraction from Con-A–Sepharose was chromatographed on Sephacryl S-300, half of the total IGF-II was found in the fraction of M_r 70000–120000. This is consistent with previous work on bound fractions from Con-A–Sepharose chromatography of Sephacryl S-300 fractions, which indicated that most of the active acid-dissociable complex was present in fractions of M_r 35000–200000, co-eluting with NSILA-P, which however is also found in fractions of M_r 200000–600000 (Cornell *et al.*, 1985). Hence it can be assumed that the bound fractions contain both the active IGF-II-rich complex together with acid-stable NSILA-P, and the preliminary results of acidic gel-permeation chromatography support this conclusion.

The discovery of an active IGF-II complex in serum further compounds the problem of having to explain why a high degree of hypoglycaemia does not exist in the normal population. Several factors may influence the amount of NSILA available to the relevant cells, as follows.

(*a*) The amount of NSILA *in vivo* may not be as high as the amounts indicated by the bioassay because of the different conditions imposed. In the isolated rat adipocyte assay, conditions are perhaps sufficiently different from the physiological state to obtain full activity from both the acid-stable complex (NSILA-P) and the active acid-dissociable complex.

(*b*) The regulation of activity of the active acid-dissociable complex (but not the acid-stable NSILA-P) by an inhibitor in serum (Herington & Kuffer, 1981; Phillips *et al.*, 1984) may be considerably greater *in vivo* compared with *in vitro*. Other inhibitors are known to be present on cell surfaces, e.g. a glycoprotein inhibitor has been isolated from the cell surface of cultured chicken embryo fibroblasts which exerted growth-inhibitory activity on these fibroblasts. It was shown that some of the glycoproteins present bound specifically to insulin or insulin-like factors in serum (Yaoi, 1982).

(*c*) The comparatively higher molecular size of the active complex may be sufficient to prevent its passage across capillaries and thus it may not be present in sufficient concentration at the cell membrane to cause insulin-like effects (Zapf *et al.*, 1975; Kaufmann *et al.*, 1977).

There is evidence to suggest that IGF-II may be a foetal growth factor (Adams *et al.*, 1983). Whilst this may be true, the fact that IGF-II levels remain at high levels (3–4 times that of IGF-I) throughout adulthood is suggestive of another role. The results of this work do not rule out a metabolic role for IGF-II, where the high M_r of the active form, perhaps in concert with an inhibitor, provide the restraints necessary for very fine regulation.

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