PARTIAL PURIFICATION AND CHARACTERIZATION OF A BINDING PROTEIN FOR INSULIN-LIKE ACTIVITY (ILAs) IN HUMAN AMNIOTIC FLUID: A POSSIBLE INHIBITOR OF INSULIN-LIKE ACTIVITY

By

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ABSTRACT

An insulin radioreceptor assay (INS-RRA) and an insulin-like activity radioreceptor assay (ILAs-RRA) have been utilized to partially purify and characterize a protein from human amniotic fluid with ILAs-RRA reactivity. An acid-ethanol soluble protein with an apparent molecular weight of 34 500 daltons by calibrated Sephadex chromatography and an isoelectric point (pI) of 4.7 accounts for all of the ILAs-RRA reactivity present in human amniotic fluid. Since this protein has been found to be a binding protein for ILAs, but not for insulin, it has been termed amniotic fluid binding protein or AFBP. AFBP is reactive in a non-parallel manner in the ILAs-RRA and totally inactive in the INS-RRA. The activity of AFBP in the ILAs-RRA is thus to the competition of AFBP with the

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placental membrane receptor for the $^{125}$ILAs tracer employed in the ILAs-RRA.

AFBP inhibits the activity of added ILAs, but not of added insulin, in the INS-RRA, presumably by binding ILAs, while being inactive itself. In two biological assays studied to date, the rat epididymal fat pad assay and the rabbit chondrocyte sulphation assay, AFBP also inhibits the activity of added ILAs. These observations raise the possibility that binding protein(s) for insulin-like peptides may function as inhibitors of their bioactivity in different physiologic and pathologic states. The relation of AFBP to binding protein(s) in human plasma remains to be clarified.

The most rapid growth in human cell number and cell size occurs during the intra-uterine and immediate post-natal periods of life (Cheek 1968; Winik 1971; Winik & Noble 1965). The factors regulating intra-uterine growth are poorly understood. Growth hormone (GH) from the maternal or foetal pituitary may not be rate limiting (Blizzard & Alberts 1956; Chez et al. 1970; Jost 1947; Laron & Pertzelan 1969; Rimoin et al. 1966; Van Assche et al. 1969; Wells 1947) although definitive human foetal studies of pituitary secretion of GH and of prolactin (PRL) in vivo have not been performed.

The importance of other factors, including insulin and somatomedin (SM) (Chochinov & Daughaday 1976; Daughaday et al. 1972; Van Wyk et al. 1974; Van Wyk & Underwood 1975), on somatic and skeletal growth in the foetus remains speculative. However, the significantly retarded birth length observed in infants with Laron dwarfism (Laron & Pertzelan 1969; Laron et al. 1968) suggests that somatomedin may affect foetal growth. The hormones or factors which promote synthesis and secretion of somatomedin in the foetus are unknown. Somatomedin bioactivity is low in amniotic fluid (Bala et al. 1978). Somatomedin bioactivity (Svan et al. 1977; Tato et al. 1975) and somatomedin reactivity by radioreceptor assay (RRA) or radioimmunoassay (RIA) is also decreased in cord blood (Furlanetto et al. 1977a,b; Heinrich et al. 1978).

Chochinov et al. (1977) have described a protein in mid-term human amniotic fluid that reacted in the somatomedin C RRA by binding labelled SM C and that was capable of inhibiting the stimulation of thymidine uptake induced by human serum and by rat SM in human fibroblast cultures. The significance of this amniotic fluid binding protein in relation to foetal growth is uncertain. In this report we confirm and extend the observations of Chochinov et al. (1977). Our studies have been carried out with an insulin-like somatomedin peptide (ILAs) that we have partially purified in our own laboratory (Posner et al. 1977, 1978). Using a radioreceptor assay for this peptide we have been able to characterize and partially purify a protein from human amniotic fluid (AFBP) that specifically binds ILAs and inhibits its bioactivity in vitro.
MATERIALS AND METHODS

Radioceptor assay (RRA) for insulin (INS) and insulin-like activity (ILAs)

The RRA procedure was identical to the method employed for INS and ILAs as described previously (Posner et al. 1977, 1978). AFBP is reactive in the ILAs-RRA, but because of non-parallel displacement quantitative results (reported as ng equivalents of porcine insulin (1 ng equivalent equals 25 µU insulin)) should be taken as an approximation only.

In order to study the binding of AFBP to the placental receptor, the ILAs-RRA was modified as follows: in each assay tube 0.1 ml of the placental membrane suspension and 0.1 ml of ILAs standards or AFBP were combined in a total volume of 0.5 ml of assay buffer (25 mm Tris, 10 mm MgCl₂, 0.1% BSA (Sigma), pH 7.4) and pre-incubated at 4°C for 2 h with continuous agitation. The incubation was stopped with the addition of 3 ml of ice-cold assay buffer. The tubes were then centrifuged at 750 x g for 30 min, the supernatant was decanted, and the membrane pellet was rinsed once with 3 ml of ice cold buffer. The membrane pellet was then incubated with [¹²⁵I]ILAs (20 000 cpm) at 4°C for 2 h with continuous agitation as in the usual assay conditions. Following the addition of 3 ml of ice cold assay buffer, membrane bound radioactivity was separated by centrifugation and counted in a gamma spectrometer (Packard Model 5120, efficiency of 40%).

Purification of AFBP

a) Amniotic fluid (AF). - Amniotic fluid was collected per vaginam at the time of rupture of the membranes from normal pregnant women at term (AF₉₅-TERM). Early gestational AF (AF₉₅-TERM) was obtained for various diagnostic purposes by amniocentesis at a gestational age of 14–20 weeks. Any sample showing more than minimal blood contamination was discarded.

The samples were pooled, frozen and stored at −20°C until processed. Upon thawing the pooled fluid was cleared of particulate matter by filtration through cheese cloth and then through coarse filter paper and finally lyophilized and stored at 4°C until used.

b) Acid-ethanol extraction. - Lyophilized pooled AF powder was extracted with cold acid-ethanol (75% ethanol (v/v) – 0.19 N HCl, pre-cooled to 4°C). Lyophilized AF powder was homogenized in cold acid-ethanol (0.1 g/ml) in a Waring blender for 4 min and stirred at room temperature for 30 min. The suspension was then centrifuged at 30 000 x g for 30 min. The supernatant was dialyzed (Spectropor-3) against distilled H₂O for 48 h and then against 0.01 N HCl for 24 h. The dialysate was frozen and lyophilized.

c) Column chromatography. - Gel filtration of lyophilized dialysate was performed on columns of Sephadex G-150 (Pharmacia Fine Chemicals, Inc., Uppsala, Sweden) (column size 5 x 100 cm) at 4°C. The following buffer systems were used for purification: 0.01 M NH₄HCO₃, pH 7.4 and 0.01 N HCl, 0.1% bovine serum albumin (BSA) (Sigma) pH 2.2. The elution volume of the amniotic fluid binding protein (AFBP) was determined by radioreceptor assay for ILAs (vide supra) on the eluted fractions. The void volume (V₀) and the total volume (Vₜ) of the column were determined with ¹²⁵I-labelled bovine thyroglobulin and ¹²⁵I, respectively. Column fractions with the highest reactivity in the ILAs RRA (Kd 0.4 to 0.5) were pooled and lyophilized.
d) Preparative isoelectric focusing. – Preparative isoelectric focusing of AFBP obtained following Sephadex chromatography was carried out in a sucrose gradient (5–50 %) stabilized column (LKB 440 ml) at 4°C. A 2 % solution of Ampholine (pH 3–6) was employed. The anode solution consisted of 0.25 M H$_2$SO$_4$ in 80 % (v/v) sucrose. The cathode solution was 0.25 M NaOH in water. After 72 h the column was emptied from below with continuous monitoring of absorbance at 280 nm. Eluate fractions were grouped and pooled according to pH and extensively dialyzed first against distilled water for 48 h and then against 0.01 M HCl for 24 h. Fractions with the highest specific activity (pH range 4.7 to 4.9) were lyophilized, re-dissolved in 0.01 M HCl, and re-chromatographed on Sephadex G-150 in the same buffer (column size 2.2 × 40 cm).

e) Iodination. – AFBP was iodinated by a modification of the chloramine T method. Partially purified material (5–10 µg protein) was added to 0.5 M phosphate buffer, pH 7.4 (10 µl), 1.0 mCi Na$^{125}$I (10–20 µl) and 40 µg of chloramine T (25 µl). The reaction was terminated after 30–45 seconds by adding 250 µg of sodium metabisulphite (100 µl), followed by 100 µg of KI (100 µl). This mixture was chromatographed directly on a Sephadex G-150 column (2.2 × 40 cm) at 4°C using 0.01 M HCl as eluting buffer. Only fractions eluting between a Kd of 0.4–0.5 were used for further studies.

Analytical procedures

Electrophoresis. – Analytical (disc) polyacrylamide gel electrophoresis (PAGE) was carried out using a 10 % acrylamide gel system. a 3 % acrylamide concentrating gel and a continuous buffer system of Tris-glycine-HCl, pH 8.3. SDS-polyacrylamide disc gel electrophoresis was performed as described by Laemmli (1970).

Isoelectric focusing. – Analytical isoelectric focusing was performed at 4°C in 7.5 % polyacrylamide, 5 % glycerol and 7 µM riboflavin-5'-phosphate containing 2 % Ampholine (biolyte, Biorad, Cal.) pH range 3–6. The cathode solution consisted of 0.02 M Ca(OH)$_2$ and 0.04 M NaOH and the anode solution was 0.06 M H$_2$SO$_4$. Electrophoresis was carried out at 200 V for 18–22 h. The gels were subsequently frozen on dry ice and sectioned into fractions using a Mickle gel slicer. Slices (3 mm) were incubated with shaking for 16 h in 0.5 ml distilled water after which pH, IIAs RRA reactivity, or radioactivity of $^{125}$I-labelled material was determined.

Molecular weight estimation. – The molecular weight of AFBP was estimated by gel filtration on a Sephadex G-150 column (2.2 × 40 cm) calibrated in 0.1 M ammonium acetate, pH 7.4. As molecular weight standards the following protein and peptides were chromatographed: aldolase 158 000; ovalbumin 45 000; chymotrypsinogen A 25 000; ribonuclease (RNase) 13 700; parathyroid hormone 9500; calcitonin 3400.

Bioassays. – Bioactivity of AFBP alone and AFBP in combination with IIA s was studied in chondrocyte cultures prepared from rabbit growth plate and articular chondrocytes (Corvol et al. 1978) as well as in the epididymal fat pad assay as described previously (Posner et al. 1978).

Protein concentration was determined by the method of Lowry et al. (1951) with bovine serum albumin (BSA) as standard, or by optical density at 280 nm.

Statistical analysis were performed by the Student’s t-test.
RESULTS

Purification

Amniotic fluid binding protein (AFBP) was partially purified from batches of term and pre-term AF by the following sequence of procedures: acid ethanol extraction, gel filtration on Sephadex G-150 (repeated once), preparative isoelectric focusing, and re-chromatography on Sephadex G-150. These procedures resulted in the isolation of samples of AFBP which had been purified from amniotic fluid about 825-fold (Table 1). Purification and recovery results should be taken as an approximation only since the dilution curves in the ILAs-RRA of untreated amniotic fluid and AFBP at various stages of purity were not parallel to the ILAs standards. These relationships are more easily seen after logit-log transformation (Fig. 1). Because of this AFBP was assayed at the same protein concentration at each step of purification.

Pre-term AF contained 7–10 times more ILAs RRA displacing activity than term AF and was usually utilized as starting material. Lyophilized AF was extracted with acid ethanol (0.1 g dry weight/ml) as described under Methods. This resulted in an approximately 7-fold purification and removal of almost 90% of protein. Lyophilized dialysate powder was dissolved in 0.01 M NH₄HCO₃ and then chromatographed on Sephadex G-150. The peak RRA activity eluted at a Kd value of 0.4–0.5 and peak reactive fractions were pooled and lyophilized. This step resulted in only slight purification with a low recovery. Subsequently it was found that higher recovery could be obtained when AFBP was kept at acid pH. Therefore material obtained as above was re-chromatographed on Sephadex G-150 (column size 3.75 x 65 cm) in 0.01 N HCl, pH 2.2.

Table 1.

<table>
<thead>
<tr>
<th>Step</th>
<th>Protein (Lowry) (mg)</th>
<th>RRA (ng equiv./ml)</th>
<th>Specific activity (ng equiv./mg)</th>
<th>Recovery (per cent)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>AF 1250 ml</td>
<td>7875</td>
<td>1025 000</td>
<td>130</td>
</tr>
<tr>
<td>2.</td>
<td>Acid ethanol extrac-</td>
<td>990</td>
<td>816 750</td>
<td>825</td>
</tr>
<tr>
<td></td>
<td>tion and dialysis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.</td>
<td>Sephadex G-150</td>
<td>230</td>
<td>263 120</td>
<td>1150</td>
</tr>
<tr>
<td></td>
<td>Repeated</td>
<td>73</td>
<td>232 960</td>
<td>3200</td>
</tr>
<tr>
<td>4.</td>
<td>Isoelectric focusing</td>
<td>8.7</td>
<td>139 776</td>
<td>16 000</td>
</tr>
<tr>
<td>5.</td>
<td>Sephadex G-150</td>
<td>0.78</td>
<td>83 865</td>
<td>107 000</td>
</tr>
</tbody>
</table>

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Purification was now about 3-fold with a recovery of 89\% and RRA reactive fractions eluted at Kd 0.4–0.5, similar to the elution profile at neutral pH. Column fractions with highest activity from several chromatograms were pooled and lyophilized. In the next step material obtained as above was subjected to preparative isoelectric focusing (see Fig. 2 and legend for details). This procedure resulted in a further 5-fold purification and recovery was estimated at 59\%. The fractions with the highest specific activity (S. A.) in the pH range 4.7–4.9 were dialyzed, lyophilized and re-dissolved in 0.01 \text{n} HCl and chromatographed on Sephadex G-150 in the same buffer (column size 2.2 \times 40 \text{ cm}). A protein peak at the Kd value of albumin was clearly separated from RRA reactive material (data not shown). This procedure resulted in a further 7-fold purification with a recovery of 60\%.

Table 1 summarizes the results obtained throughout the entire sequence of procedures used for the isolation and partial purification of AFBP. The final recovery of RRA activity was estimated at about 6\%. The apparent purification was 825-fold. Analytical polyacrylamide disc gel electrophoresis in SDS of the final product showed one major protein band with an \( R_F \) of 0.44 (Fig. 3).
Preparative isoelectric focusing. AFBP partially purified from term AF by acid-ethanol extraction and Sephadex G-150 chromatography (2X) with S. A. of 335 ng equiv./mg protein was dissolved in 18 ml H$_2$O (total amount of protein was 150 mg) and applied to a sucrose stabilized (5-50 %) column (LKB 440 ml) at 4°C. Peak RRA activity in the eluted fractions measured after extensive dialysis was seen at pH 4.7-4.9. Bars represent S. A. in ng equiv./mg prot.

- - pH gradient.
- - protein measured by OD at 280.

Analytical gel electrophoresis in polyacrylamide gel (7.5 %) with SDS.

a) The main protein band of pre-term amniotic fluid (albumin) had an $R_F$ of 0.44.

b) Partially purified AFBP (100 ng equiv.) revealed only one protein band at $R_F = 0.44$.  

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Molecular weight

The molecular weight determination of AFBP was based on gel filtration on a standardized column of Sephadex G-150. In many successive runs of partially purified AFBP in neutral pH buffers, the Kd values were not significantly different and the combined Kd value for these was $0.435 \pm 0.09$ (mean ± sem, n = 7). The range was from 0.403 to 0.472. This provides a molecular weight estimate of 34,500 daltons.

Isoelectric point

As indicated in Fig. 2, during preparative isoelectric focusing the main RRA activity eluted at a pH between 4.7–4.9. When AFBP labelled with $^{125}$I as outlined under Methods was subjected to isoelectric focusing in polyacrylamide gels a major peak was seen between pH 4.6–4.9. A minor peak was found between 4.2–4.5 but it probably represents tailing of damaged tracer products as RRA activity was eluted only at pH 4.7–4.9 (not shown).

The nature of the interaction between AFBP and ILAs was defined by several different kinds of studies.

![AFBP INTERFERENCE in INS RRA](image)

Fig. 4.

Results of the assay of ILAs or of a mixture of ILAs and AFBP in the INS-RRA (after logit-log transformation). In the mixtures, concentrations of ILAs in the sample can be read off the x axis and concentrations of AFBP (in ng equiv./ml) are indicated along the corresponding regression line.

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Table 2.
Binding of [125I]ILAs to placental membranes after pre-incubation with ILAs or AFBP.

<table>
<thead>
<tr>
<th>Peptide added*</th>
<th>B-N</th>
<th>B_o-N</th>
</tr>
</thead>
<tbody>
<tr>
<td>ILAs</td>
<td>0.7621</td>
<td></td>
</tr>
<tr>
<td>0.25</td>
<td>0.7380</td>
<td></td>
</tr>
<tr>
<td>1.0</td>
<td>0.6294</td>
<td></td>
</tr>
<tr>
<td>2.5</td>
<td>0.5638</td>
<td></td>
</tr>
<tr>
<td>4.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AFBP</td>
<td>0.9900</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>0.9903</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>0.9952</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>1.050</td>
<td></td>
</tr>
<tr>
<td>90</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Final concentration in ng equiv. of porcine insulin (see Materials and Methods).

Epididymal fat pad assay. The stimulatory effect of insulin on the incorporation of [14C]glucose into fatty acids is expressed as 14C cpm/mg tissue. Incubating AFBP (S. A. approximately 1000 ng equiv./mg prot.) at a concentration of 100 ng equiv./flask has no stimulatory effect. This amount added to ILAs (66 ng equiv./flask) decreased the stimulatory effect of ILAs alone significantly (P < 0.005).

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a) Radioreceptor assays. – To exclude the possibility that the RRA reactivity of AFBP is based in part on direct binding of AFBP to the receptor, the ILAs-RRA was modified as outlined under Methods. Ninety ng equiv. AFBP (S.A. 1250 ng equiv./mg prot.) pre-incubated with placental membranes did not inhibit \(^{[125]I}\)ILAs binding to the receptor whereas 4 ng equiv. ILAs significantly decreased specific binding (Table 2). In addition, \(^{[125]I}\)AFBP incubated with receptor under usual assay conditions for 2, 4, 6 and 24 h did not reveal any specific binding. Finally, when a mixture of ILAs and AFBP was assayed in the INS-RRA, one observed that progressively higher concentrations of AFBP inhibited the activity of ILAs in the INS-RRA, although when assayed alone in the INS-RRA, AFBP was non-reactive (see Fig. 4 and legend for details).

![Sulfation Assay in Rabbit Chondrocytes](image)

**Fig. 6.**
Monolayer chondrocyte cell cultures prepared from rabbit growth plate and articulate chondrocytes were incubated for 20 h in Dulbecco's medium (Flobio, Courbevoie, France) without fetal calf serum. This medium was then replaced with sulphate-free Dulbecco's modified medium (MgCl\(_2\) for MgSO\(_4\)) to which was added 1.5 \(\mu\)Ci/ml Na\(_2\)\(^{35}\)SO\(_4\) in the presence of different concentrations of ILAs, AFBP (S.A. 1250 ng equiv./mg prot.), or a mixture of both.
Partially purified AFBP (S.A. 1250 ng equiv./mg prot.) was chromatographed on Sephadex G-150 (column dimensions 0.8 x 50 cm) at 4°C. 0.3 ml fractions were collected.

- RRA activity.
- Radioactivity.

a) RRA activity was measured after approximately 90 ng equiv. AFBP was dissolved and run in 25 mM Tris-HCl, 0.1% BSA buffer (pH 7.4). Pre-incubation with 150 000 cpm [125I]ILAs at 4°C for 1 h in the same buffer resulted in superimposable peaks.

b) Same quantities as in (a) but run in 0.01 N HCl (pH 2.2) shows reversal of binding.

c) In addition to AFBP and [125I]ILAs as above, 12 ng equiv. unlabelled ILAs was added to the pre-incubation mixture with buffer: 25 mM Tris-HCl, 0.1% BSA, pH 7.4.

d) Idem except 120 ng equiv. ILAs was added in the pre-incubation step.

b) Bioassays. - AFBP significantly inhibited the stimulatory effect of ILAs on [14C]glucose incorporation into fatty acids of rat adipose tissue (see Fig. 5 and legend). The increase of [35S]sulphate incorporation into purified proteoglycans of rabbit articular chondrocytes in monolayer culture produced by ILAs was completely blocked by AFBP (Fig. 6).

c) Sephadex chromatography. - Further evidence for the role of AFBP as a binding protein for ILAs is presented in Fig. 7. AFBP pre-incubated with [125I]ILAs and chromatographed on Sephadex G-150 at neutral pH resulted in a radioactive peak superimposable on the peak of AFBP as determined by ILAs RRA (Fig. 7 a). No binding of ILAs occurred at pH 2.2 (Fig. 7 b).
[125I]ILAs and AFBP were pre-incubated in the presence of unlabelled ILAs, some radioactivity was now eluted at the Kd of ILAs (0.7). Increasing the concentration of unlabelled ILAs 10-fold resulted in most of the radioactivity being eluted at a Kd of 0.7. Unlabelled insulin (500 ng) added to the pre-incubation mixture did not displace [125I]ILAs from AFBP.

**Discussion**

Endogenous plasma ILAs is bound to large molecular weight proteins, and this binding is easily reversed by exposure to acid pH (Guyda et al. 1977). Gel filtration of amniotic fluid in 0.01 N HCl or in 1.0 M acetic acid (two procedures which have been uniformly effective in dissociating ILAs from binding proteins in plasma) have failed to demonstrate the presence of small molecular weight ILAs in human amniotic fluid. When lyophilized amniotic fluid powder was re-dissolved in 1/20th the original volume and chromatographed in 0.01 N HCl, thereby concentration 20-fold, no ILAs-RRA reactivity was observed in fractions where ILAs would be expected. When incubated and chromatographed in 8 M urea, a procedure known to dissociate protein complexes, the ILAs-RRA reactivity, although diminished, was again recovered only in the large molecular weight region of the column. ILAs itself was stable in 8 M urea. It would therefore seem that the small molecular weight ILAs was absent from amniotic fluid and that all the ILAs-RRA activity could be accounted for by a moderately large (approximate M.W. 34 x 10^3 daltons) acid protein (or proteins) we have termed AFBP. AFBP is heat labile since more than 90 % of its RRA reactivity was lost by heating to 100°C for 60 min.

The AFBP identified in amniotic fluid does not appear to be a protein-ILAs complex but it does display specific ILAs binding activity (Fig. 7). Binding of [125I]ILAs by AFBP was pH dependent, being dissociated at pH 2.2. In addition, a dose-dependent inhibition of [125I]ILAs binding was observed on adding various amounts of unlabelled ILAs. No inhibition of [125I]ILAs binding by unlabelled insulin was observed under identical conditions thus demonstrating the specificity of the AFBP-ILAs interaction.

To insure that AFBP did not inhibit binding of [125I]ILAs to its placental receptor by competing for the binding sites, placental membrane binding of [125I]ILAs was tested after prior exposure to AFBP. Binding of [125I]ILAs was not inhibited by prior exposure of the membrane to AFBP. It therefore would appear that the ILAs-RRA activity of this protein was due to its ability to bind labelled ILAs thus making it unavailable to the placental receptor.

Very little has been published on somatomedins or NSILAs in amniotic fluid. Bala et al. (1978) have reported that somatomedin, as measured by an in vitro hypophysectomized rat cartilage assay, was heterogeneous and consists of at least three components. The major fraction, accounting for 86% of the
total activity, had an apparent molecular weight of more than 50,000 and did not dissociate to smaller molecules in 1% formic acid. The SM bioactivity was very low at midterm.

As our work was in progress, Chochinov et al. (1977) reported the results of their investigation of an amniotic fluid somatomedin binding protein using a SM C-RRA. Their results closely parallel ours, as to molecular weight, isoelectric point, non-dissociation into smaller components, and presence of the large molecular protein SM as the only detectable reactivity in the SM C-RRA. Direct comparisons between our AFBP and Chochinov’s SM C-RRA reactive amniotic fluid protein (kindly provided by Chochinov) in our ILAs RRA indicated similar behaviour.

AFBP does not cross-react in the INS-RRA, even at a concentration of 500 ng/ equiv. ml. and one would predict that it would therefore be inactive in an insulin bioassay system. Such indeed is the case, since our material was not active in the epididymal fat pad assay. Furthermore, it inhibited ILAs activity in this bioassay system, presumably by binding ILAs. In addition, complete inhibition of sulphahtion activity by ILAs was observed in cultured rabbit chondrocytes. These data suggest that bound ILAs was not active with insulin tissue receptors and are in keeping with the observations that "bound insulin" or NSILAs is not physiologically active in vivo (Posner et al. 1968) or in vitro (Meuli et al. 1978).

Salmon (1972, 1975) has reported the presence of a somatomedin inhibitor in sera of hypophysectomized or starved rats and has postulated a physiological role for this inhibitor in limiting anabolic events, possibly to a greater extent than that resulting from the decrease of growth hormone release. Phillips (1978) has also described a somatomedin inhibitor in diabetic rat sera that has a molecular weight between 10 to 60 |x 10^3 daltons by Sephadex chromatography. These inhibitors could account for the dichotomy between growth hormone and somatomedin levels in human clinical conditions such as the emotional deprivation syndrome or Kwashiorkor (Van den Brande & Du Caju 1974; Van den Brande et al. 1975). The relationship between AFBP and these other SM inhibitors of plasma remains to be clarified. The development of a specific RIA for AFBP should facilitate its study (Drop et al. 1978).

REFERENCES


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