Human Amniotic Fluid Prolactin. Purification by Affinity Chromatography and Amino-Terminal Sequence†

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ABSTRACT: Prolactin was purified from human amniotic fluid by affinity chromatography. The preparation immunologically and electrophoretically was similar to purified human pituitary prolactin. Studies of the amino acid composition of pituitary and amniotic fluid prolactin confirmed the similarity of the prolactins. The amino acid sequences of the N-terminal 25 residues of the two prolactin preparations appear identical. Cystine residues which are found at positions 4 and 11 in human pituitary prolactin were presumed to occupy the same positions in amniotic fluid prolactin.

It is now firmly established that prolactin exists as a distinct hormone in man: two groups of investigators have purified the hormone from human pituitary glands (Lewis *et al.*, 1971; Hwang *et al.*, 1972) and its primary structure has been partly elucidated (Niall *et al.*, 1973). While using radioimmunoassays to study prolactin concentrations in human biological fluids, we noted that human as well as monkey amniotic fluid contained very high concentrations of prolactin (Friesen *et al.*, 1972). We now report the purification of amniotic fluid prolactin by a simple procedure using affinity chromatography and the amino acid sequence of the N-terminal 25 residues. The application of this isolation method on a larger scale should make it feasible to generate considerable amounts of prolactin from amniotic fluid for further chemical and biological studies. A previous attempt at purifying amniotic fluid prolactin using chemical procedures resulted in a partially purified preparation (Hwang *et al.*, 1974).

Materials and Methods

Materials. The amniotic fluid employed in this study was obtained during the course of therapeutic abortions before the 20th week of gestation and stored frozen with 0.02% sodium azide as preservative. Earlier observations (Friesen *et al.*, 1972) showed that amniotic fluid from early pregnancy contained higher concentrations of prolactin than amniotic fluid obtained at term. Human pituitary prolactin was purified from frozen glands as previously described (Hwang *et al.*, 1972). Antisera against human pituitary prolactin were generated in rabbits by conventional methods.

Preparation of Immunoadsorbents. The purification and coupling to Sepharose of antibodies against human pituitary prolactin were carried out by the procedure reported previously for the purification of monkey prolactin (Guyda and Friesen, 1971). Briefly, 5 mg of human pituitary prolactin was coupled by the cyanogen bromide method to 10 ml of Sepharose 4B which was then packed into a column. Rabbit antiserum (200 ml) against human pituitary prolactin, capable of binding a total of 4 mg of prolactin, was diluted to 800 ml with 0.1 M Tris (pH 8.5) and passed once through the column of anti-human prolactin Sepharose at a flow rate of about 1 ml/min at room temperature. More than 95% of the specific antibody against human prolactin was adsorbed. Elution of the specific antibody was carried out with 4 M NaSCN in phosphate–saline buffer (pH 7.4). After desalting by gel filtration on Sephadex G-25 with 0.01 M NH_4HCO_3 as buffer the antibody fraction was coupled to 10 ml of Sepharose 4B.

Radioimmunoassays for prolactin were carried out as described (Hwang *et al.*, 1971). *Protein* concentrations were estimated as before (Hwang *et al.*, 1972).

Purification Procedure. All operations were carried out at room temperature. Amniotic fluid (2 l.) was filtered through a Buchner funnel (medium) and the pH was adjusted to 7.4 by the addition of 200 ml of 1 M Tris followed by the appropriate amount of 5 N HCl. The amniotic fluid was then passed once through the anti-prolactin Sepharose column at a flow rate of about 1 ml/min. The column was washed with 500 ml of 0.1 M Tris (pH 7.4) and then eluted with 4 M sodium thiocyanate at a flow rate of 0.5 ml/min; 4-ml fractions were collected. The protein concentrations were determined by reading the absorbance at 278 nm and appropriate tubes were pooled and immediately applied to a Sephadex G-100 column (2 × 90 cm) equilibrated with 0.01 M NH_4HCO_3. Fractions collected were monitored for protein and prolactin. Appropriate tubes were pooled and concentrated by ultrafiltration (Amicon UM 10) and lyophilized.

Electrophoresis on polyacrylamide gel was performed according to Davis (1964).

Immunological Studies. Purified amniotic fluid prolactin was iodinated by the chloramine-T method of Hunter and Greenwood (1962). Excess antiserum to human pituitary prolactin was added to determine the proportion of radioactivity specifically precipitable by the antiserum, using the double antibody method with normal rabbit serum as control.

The immunological similarity of pituitary and amniotic fluid prolactins was further tested by comparing the slopes of their respective displacement curves in the human prolactin radioimmunoassay.

Amino Acid Analysis. Pituitary and amniotic fluid prolactin was hydrolyzed by constant boiling in 5.7 N HCl for 24 hr. Amino acid analysis of the hydrolysates was performed on a Beckman Model 121 amino acid analyzer (Beckman Instruments, Palo Alto, Calif.).

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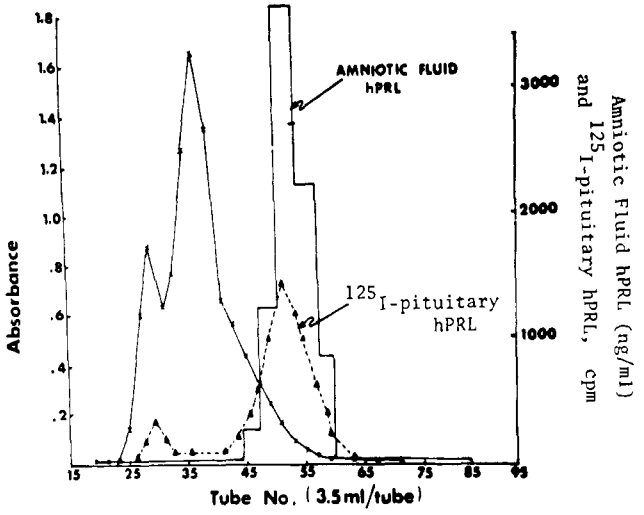

FIGURE 1: Sephadex G-100 fractionation of crude amniotic fluid. Flow rate, 1 ml/min. A small amount of ^{125}I pituitary hPRL was added as a marker to the amniotic fluid sample prior to gel filtration.

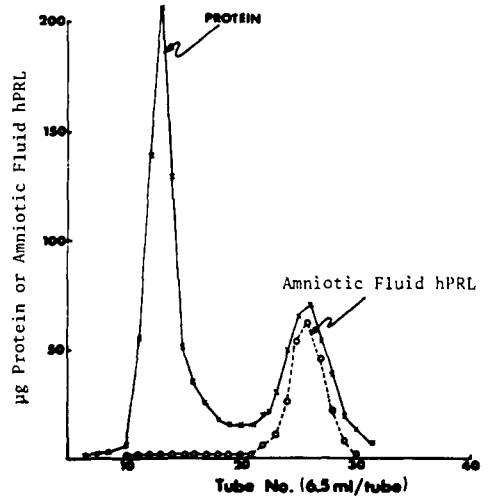

FIGURE 2: Distribution of protein and amniotic fluid after Sephadex G-100 fractionation of material eluted from anti-prolactin Sepharose. The amniotic fluid hPRL was measured by radioimmunoassay.

Automated Sequence Analysis. Purified amniotic fluid prolactin (1 mg) was dissolved in 20% acetic acid and transferred to the reaction cell of a Beckman Model 890C Sequencer. Automated sequence analysis was carried out by a modification (Jacobs *et al.*, 1973) of the procedure of Edman and Begg (1967). Two coupling stages were performed in each cycle of degradation. The first coupling employed high specific activity [^{35}S]phenyl isothiocyanate (0.5%, v/v, in *n*-heptane; Amersham-Searle, Chicago). After extractions with benzene and ethyl acetate, and a vacuum stage, a second coupling was carried out with nonradioactive phenyl isothiocyanate (5%, v/v in *n*-heptane). This procedure results in high specific activity labeling of the amino acid derivatives; the second coupling at the higher reagent concentration is necessary to ensure complete reaction at each cycle. Nonradioactive sequenator reagents were obtained from Beckman Instruments (Palo Alto, Calif.).

The thiazolinone amino acid derivatives cleaved at each cycle of degradation were converted to the more stable phenylthiohydantoinyl derivatives by the procedure of Ilse and Edman (1963). Aliquots of each organic phase were analyzed quantitatively by gas–liquid chromatography (Pisano and Bronzert, 1969; Niall *et al.*, 1973). The remainder of the organic phase sample was mixed with carrier phenylthiohydantoin amino acids and analyzed by two-dimensional thin-layer chromatography on silica gel plates using solvent systems based upon those described earlier by Brenner *et al.* (1962) and by Edman (1970). Radioactive spots were localized by overnight autoradiography, and scraped and counted in a Packard liquid scintillation counter to achieve quantitation. Aqueous phase samples, which contain the phenylthiohydantoinyl derivatives of arginine and histidine, were identified by one-dimensional thin-layer chromatography according to the method of Inagami (1973), and quantitated by radioactive counting as described above.

Results

During the course of this study, we have noted no appreciable loss of the immunoreactive prolactin in the amniotic fluid after thawing for periods as long as 1 week when the amniotic fluid was kept at 4° in the presence of 0.02% sodium azide. After 1 week of storage, the prolactin concentration was reduced only slightly from the starting concentration of 3.7–3.5 mg of amniotic fluid prolactin/l. At room temperature, 22°, prolactin was stable up to 24 hr but its stability at this temperature for longer periods than 24 hr has not been tested.

Figure 1 shows the distribution of protein and prolactin when a sample of amniotic fluid was fractionated on Sephadex G-100 in 0.01 M NH_4HCO_3 (2 × 90 cm). It is clear that prolactin was detected in the region where labeled human pituitary prolactin emerged, indicating that amniotic fluid prolactin is similar in molecular size to pituitary prolactin.

Figure 2 shows the distribution of proteins and prolactin when the material eluted from the anti-prolactin Sepharose column by 4 M NaSCN was fractionated on Sephadex G-100. Consistently a large protein peak was observed at the void volume where little or no prolactin could be detected. A second protein peak with a relative elution volume of about 2.5 (V_e/V_0) was associated with prolactin. In some of the fractions in this second peak, all of the protein was accounted for by prolactin as determined by radioimmunoassay; other fractions, however, contained additional proteins other than prolactin. The protein in the void volume peak probably represented nonspecifically adsorbed proteins which were not removed by the washing procedure.

In the original amniotic fluid sample the protein concentration was 6 g/l., the prolactin concentration being 3.2 mg/l. Under the condition of the affinity chromatography only about 50% of the prolactin in the amniotic fluid was adsorbed by the anti-prolactin Sepharose column. After gel filtration of the eluent on Sephadex G-100 1.46 mg of prolactin (0.73 mg/l.) was recovered in the fractions in which there was no significant contamination by other proteins. Recovery of prolactin was therefore approximately 20%.

Figure 3 shows the patterns of polyacrylamide gel electrophoresis of pituitary and amniotic fluid prolactin. At pH 9.5 while pituitary prolactin showed only two prominent bands, amniotic fluid prolactin shows two additional bands which were more anodal.

Figure 4 shows the displacement curves of human pituitary prolactin, human amniotic fluid prolactin, and crude amniotic fluid in the radioimmunoassay for human pituitary prolactin. Complete parallelism was observed with all three preparations. It is also clear that the purified amniotic fluid prolactin is

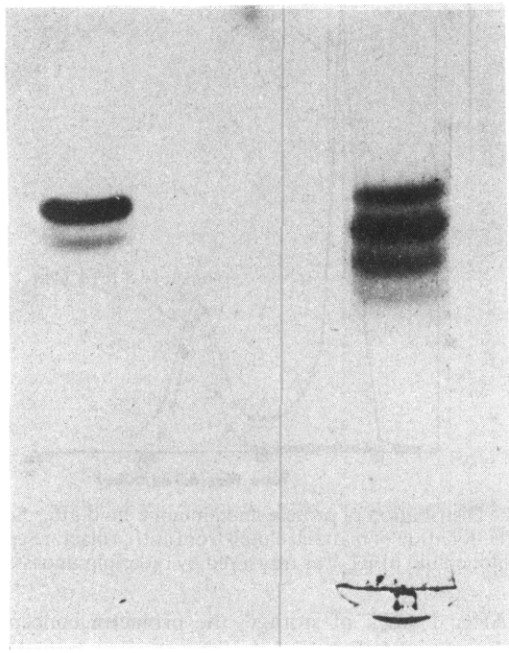

FIGURE 3: Polyacrylamide gel electrophoresis of pituitary (left) and amniotic fluid hPRL (right) at pH 9.5 and gel concentration 7.5%. The anode was at the bottom of the gel in both cases.

almost as potent as purified pituitary prolactin in the radioimmunoassay.

Figure 5 shows the inhibition curves obtained for pituitary prolactin when either labeled pituitary or labeled amniotic fluid prolactin was employed as the tracer. It is evident that the purified amniotic fluid prolactin could be used satisfactorily as a tracer in the radioimmunoassay for human prolactin. Immunoprecipitation with excess anti-pituitary prolactin antiserum showed that at least 80% of the labeled amniotic fluid prolactin could be precipitated.

The results of amino acid analyses of 24-hr hydrolysates of human pituitary prolactin and human amniotic fluid prolactin are presented in Table I. A comparison of the amino acid composition of the two prolactin preparations reveals the very close similarity of the results.

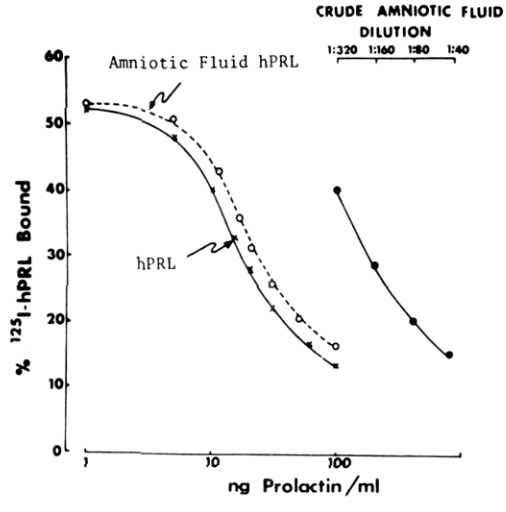

FIGURE 4: A comparison of the inhibition curves of purified human pituitary prolactin, amniotic fluid prolactin and crude amniotic fluid using a homologous radioimmunoassay for human pituitary prolactin. The assay system consisted of rabbit antibody to pituitary hPRL and ^{125}I-labeled pituitary hPRL. The results are a measure of the ability of pituitary hPRL, purified amniotic fluid hPRL, and crude amniotic fluid hPRL to inhibit binding of ^{125}I-labeled pituitary hPRL.

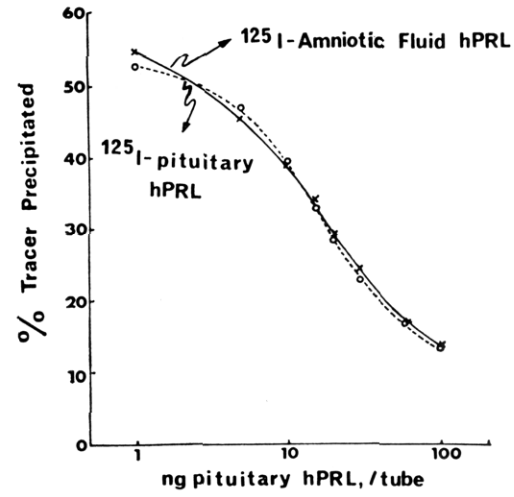

FIGURE 5: Displacement curves obtained using ^{125}I-labeled pituitary or amniotic fluid human prolactin as the tracer in radioimmunoassay for human pituitary prolactin. Rabbit antiserum to human pituitary hPRL was used.

The results of automated Edman degradation of amniotic fluid prolactin are shown in Table II. A unique amino acid sequence was clearly identified for the first 25 cycles. By this criterion, the preparation was essentially homogeneous. Contaminating polypeptides were present, but at an insignificant level as judged by quantitation by both gas–liquid chromatog-

TABLE I: Amino Acid Analysis of 24-hr Hydrolysates of Human Pituitary Prolactin and Human Amniotic Fluid Prolactin.

	Pituitary Prolactin		Amniotic Fluid Prolactin	
	Found	Taken	Found	Taken
Aspartic acid	18.6	19	20.4	20
Threonine	8.8	9	9.4	9
Serine	14.9	15[a]	15.2	15[a]
Glutamic acid	27.5	28	29.0	29
Proline	9.1	9	10.0	10
Half-cystine	5.6	6[b]		[c]
Glycine	8.5	9	10.6	11
Alanine	12.3	12	13.0	13
Valine	9.0	9[d]	9.1	9[d]
Methionine	3.5	4	3.8	4
Isoleucine	9.7	10[d]	9.2	9[d]
Leucine	24.5	25	23.5	24
Tyrosine	5.7	6	5.4	5
Phenylalanine	5.7	6	6.0	6
Tryptophan		[c]		[c]
Lysine	9.8	10	10.3	10
Histidine	7.1	7	6.8	7
Arginine	10.7	11	10.2	10

[a] Serine value after correction for losses during hydrolysis. [b] Measured as cysteic acid after performic acid oxidation. [c] Not estimated. [d] Valine and isoleucine values corrected for incomplete hydrolysis after 24 hr. [e] Values expressed as moles of amino acid per mole of prolactin, using best fit of all stable residues for normalization.

TABLE II: Phenylthiohydantoin Amino Acid Derivatives Obtained by Automated Edman Degradation of Amniotic Fluid Prolactin.[a]

Cycle No.	Residue	Yield (nmol)
1	Leu	24.0
2	Pro	4.8
3	Ile	16.2
4	*b*	
5	Pro	6.8
6	Gly	12.8
7	Gly	15.2
8	Ala	9.1
9	Ala	10.1
10	Arg	4.0
11	*b*	
12	Gln	4.8
13	Val	4.3
14	Thr	0.8
15	Leu	4.2
17	Asp	4.7
18	Leu	3.7
19	Phe	3.9
20	Asp	2.5
21	Arg	2.9
22	Ala	0.9
23	Val	1.4
24	Val	1.9
25	Leu	1.0

[a] Yields are those of the major component identified at each cycle, and have not been corrected for background or overlap. Degradation was carried out on a sample of approximately 27 nmol. [b] Cystine assumed to be present by analogy with other prolactins.

raphy (glc) and by radioactive counting of the thin-layer chromatographs (tlc). The presence of a single dominant sequence is illustrated by Figures 6 and 7. Figure 6 shows the autoradiograph obtained at cycle 1 of the degradation. A single dominant spot (the phenylthiohydantoinyl derivative of leucine) can be seen. Figure 7 shows a gas chromatographic tracing of the same sample. Again, a single dominant amino acid derivative can be seen, with low amounts of contamination by other residues.

The valine end group seen at low levels in both the glc and tlc analyses (Figures 6 and 7) may represent a minor variant of prolactin rather than a contaminant since it is also seen in about the same proportion in highly purified samples of pituitary prolactin.

No residue could be identified at cycles 4 and 11. It is extremely probable that half-cysteine residues occupy these positions, since any other naturally occurring amino acid would have been easily identifiable under the conditions of degradation. (The phenylthiohydantoinyl derivatives of cystine and cysteine are very unstable and are not normally identifiable.) Half-cystine residues are present at these positions in ovine, porcine, and human pituitary prolactins (Li *et al.*, 1970; Li, 1973; Niall *et al.*, 1973). However, definite identification of the half-cystine locations must await further studies.

When the sequence obtained for the first 25 N-terminal residues of amniotic fluid prolactin was compared to that previously established for human pituitary prolactin (Niall *et al.*, 1973), the two were found to be identical.

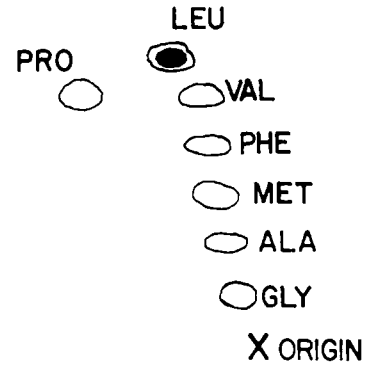

FIGURE 6: Autoradiograph (16-hr exposure) of sample from cycle 1 of automated Edman degradation of amniotic fluid prolactin, after two-dimensional thin-layer chromatography according to Brenner *et al.* (1962). On more heavily exposed autoradiographs in addition to the major component (leucine), a faint additional spot could be seen in the position of valine.

Discussion

The material we have purified from amniotic fluid using affinity chromatography appears to be very similar to, if not identical with, human pituitary prolactin. Its molecular size as indicated by gel filtration and its electrophoretic mobility

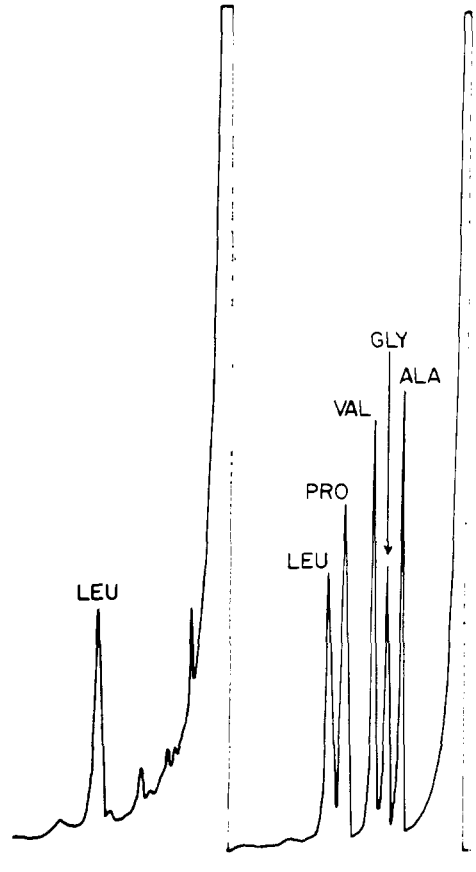

FIGURE 7: Gas chromatographic tracing of sample from cycle 1 of automated Edman degradation of amniotic fluid prolactin. (Injection shown on left.) On right is shown the tracing obtained with a mixture of amino acid phenylthiohydantoin standards. Conditions: 10% SP-400 column, 2-mm i.d. × 4 ft, isothermal injection at 190°. The small valine component in the prolactin may represent microheterogeneity of the amino-terminal residue.

upon acrylamide gel is similar to that of pituitary prolactin. It is immunologically indistinguishable from pituitary prolactin as indicated by parallel inhibition in radioimmunoassay. Its amino acid composition is very similar and the amino-terminal sequence is identical with that of pituitary prolactin for the first 25 residues. Additional support for the identity of amniotic and pituitary prolactin comes from the studies of Rodbard (1973), which indicated that these two prolactins could not be distinguished on polyacrylamide electrophoresis at a number of gel concentrations.

The data presented also suggests that the final product has been purified close to homogeneity. No significant contaminating polypeptides were detected by the N-terminal sequence analysis. This does not exclude the presence of an amino-terminal-blocked contaminant which would not be revealed by Edman degradation. However, the quantitative yields obtained in the degradation (Table II) are consistent with the amount of material being degraded, when allowance is made for some content of water of hydration, and for the usual end group recovery values for homogeneous proteins analyzed by the automated Edman procedure (Niall, 1974). Hence no more than a small proportion of amino-terminal-blocked material can be present in the preparation. In addition, the amniotic fluid prolactin is almost as active as pituitary prolactin in the radioimmunoassay, and when iodinated, 80% of the radioactivity could be specifically precipitated by antisera against pituitary prolactin. Moreover, the labeled amniotic fluid prolactin could be used satisfactorily as tracer in the radioimmunoassay for pituitary prolactin. Although polyacrylamide gel electrophoresis at pH 9.5 revealed more components in amniotic fluid prolactin than pituitary prolactin, this finding is consistent with different degrees of deamidation in the two preparations (Hwang *et al.*, 1972). In view of the chemical purity of the preparation by N-terminal analysis, the immunological potency as measured by radioimmunoassay and the 80% precipitability by anti-prolactin antiserum, it is unlikely that the two more anodal bands represent contaminant proteins.

It would appear that the purification procedure outlined is a simple and satisfactory method for obtaining amniotic fluid prolactin in a sufficiently pure state for immunological and chemical studies. Since amniotic fluid is readily available, the use of this isolation procedure on a larger scale could provide substantial quantities of highly purified prolactin. This would allow more detailed chemical, physicochemical, and immunological studies to establish beyond doubt the identity of the amniotic fluid and pituitary prolactins. If, as seems very likely, the two hormonal molecules are in fact identical, then amniotic fluid may be a practical source of purified prolactin for routine use by clinical and laboratory investigators. Though the binding capacity of the immunoadsorbants gradually decreases with repeated use, about 50% of the starting capacity is retained after six cycles.

It can be estimated that if 10% of the purified amniotic fluid prolactin is employed for the generating of antibodies, a long-term program for its purification by means of affinity chromatography can be continued without having to use purified pituitary prolactin to prepare further supplies of anti-human prolactin Sepharose. Attempts which were made to purify human amniotic fluid prolactin with anti-ovine prolactin Sepharose were unsuccessful, binding of human prolactin by this heterologous immunoabsorbant being very poor.

Neither the tissue of origin nor the physiological significance of the amniotic fluid prolactin has been established. The strikingly high levels found, and the known role of prolactin in regulating salt and water metabolism in certain fish make it tempting to propose a regulatory function, particularly if the hormone is secreted by chorionic or placental tissue. It is possible, however, that the fetal pituitary is the true source, and that accumulation of prolactin in amniotic fluid merely reflects the lack of systems capable of degrading or clearing it.

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