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QUARTERLY

Collagenous constituents of amniotic fluid*°

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The amniotic fluid (AF) was fractionated by dialysis, gel filtration and SDS/PAGE, and submitted to the assay of collagenous constituents. The collagenous character of peptides and proteins of amniotic fluid was confirmed by hydroxyproline (Hyp) assay and treatment with bacterial collagenase followed by electrophoresis and gel filtration of the digestion products. It was found that AF contains collagen degradation products but the classical method of Hyp determination described by Woessner (*Arch. Biochem. Biophys.*, 1961, 93, 440–447) gives overestimated values due to the interference with other AF components. Fractionation of AF on Sephadex G-100 column allowed to remove the interfering material and to estimate the actual Hyp content which equals to approx. 6.2 μg/ml. About 70% of Hyp was found in low molecular dialyzable products and the rest (about 30%) appears to be a constituent of non-dialyzable collagenous polypeptides of the molecular mass of about 7.9–26.3 kDa. It is suggested that such collagenous polypeptides may be the products of proteolytic conversion of collagen precursor (procollagen) into the monomeric form of this protein. No high molecular forms of collagen, corresponding to alpha-subunits, were found.

Collagen is an essential component of various organs, mainly of connective tissue. It constitutes about 30% of all proteins of the human body [1]. At least 19 genetically distinct types of this protein have been described [2]. Their common feature is the presence of triple-helical domains built of three identical or different intertwining chains, called alpha-

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Abbreviations: AF, amniotic fluid; BSA, bovine serum albumin; Hyp, hydroxyproline; NEM, *N*-ethylmaleimide; SDS, sodium dodecyl sulphate, SDS/PAGE, sodium dodecyl sulphate/polyacrylamide gel electrophoresis.

subunits [3]. This characteristic structure is due to specific amino-acid composition of collagen, with its large content of glycine (33% amino-acid residues) and cyclic imino acids (22% residues), low content of sulphuric and aromatic amino-acids, and, moreover, the presence of hydroxyproline (Hyp) and hydroxylysine, that occur but very rarely in other proteins [1]. Collagen is resistant to the action of nonspecific proteolytic enzymes but it is susceptible to the action of bacterial and tissue collagenases [1, 3].

The key role in collagen degradation is played by extracellular enzymes – matrix metalloproteinases (matrixins) [4], particularly by collagenases belonging to this group that initiate digestion of native collagen types I, II, III and act on the triple-helical region of the molecule [5]. Other matrixins are involved mainly in further cleavage of arising fragments into low molecular products, as well as in digestion of other collagens, especially that of type IV [4, 6–10].

Almost all matrixins, except the constitutive enzyme gelatinase A [6, 11], are synthesized and secreted under the influence of many cytokines and growth factors, interferones, interleukines 1α and 1β [4, 12, 13] as well as other factors, e.g. viral transformation, prostaglandin E or agents acting on the cell surface: collagen fibrils, various crystals, etc. [4]. All these factors enhance collagen degradation, that is observed for example during embryonic development, postpartum involution of the uterus, ovulation, angiogenesis, as well as in some pathological conditions [4].

Amniotic fluid, surrounding the human fetus during its intrauterinal development, is produced mainly by the amnion and fetal kidneys, and in part by umbilical cord, skin, lungs and alimentary tract of the fetus [14].

Like other body fluids, AF contains collagen metabolites. The aim of the present work was to determine hydroxyproline level and its distribution between proteins and peptides of amniotic fluid. It became also evident that non-specificity of the commonly applied method of Woessner should be taken into consideration in the estimation of Hyp content in the amniotic fluid.

MATERIALS AND METHODS

Amniotic fluid was obtained by amniocentesis, performed on clinical indications, from 33 healthy women in labour, after the normal course of gestation, delivering at term (38–42 week of gestation). Samples contaminated with blood were discarded. Fluids were centrifuged at 1000 × ***g*** for 20 min to remove cells and insoluble constituents. The supernatants were collected and stored at –20°C.

Dialysis. The samples of AF were placed in dialysis tubings (Medicell International Ltd., London, England) and dialyzed against 100 vol. of distilled water (with constant stirring), at 4°C, for 24 h. The water was changed and dialysis continued for the next 24 h.

Protein assay. Protein concentration in dialyzed AF was measured by the microbiuret method [15].

Concentration procedure. The samples of AF were lyophilized and dissolved in 50 mM NH_4HCO_3 in 1/5 or 1/10 volume of AF original.

Hydroxyproline was determined by the method of Woessner [16] after hydrolysis of proteins in 6 M HCl, at 124°C, for 18 h. To check the specificity of the colour reaction characteristic for hydroxyproline in collagen and AF hydrolysates, the absorption spectra at 500–600 nm were compared with that for hydroxyproline chromogen.

Enzymatic identification of collagenous proteins. The method described by Peterkofsky & Diegelmann [17] was applied. The reaction mixture contained 1 ml of dialyzed, 10-fold concentrated AF and 0.8 ml of highly purified bacterial collagenase (Sigma, U.S.A.) solution in 50 mM Tris/HCl buffer + 10 mM $CaCl_2$, pH 7.4, and 0.2 ml of 25 mM *N*-ethylmaleimide (NEM) as an inhibitor of non-specific proteolytic activity of collagenase.

The control sample contained 0.8 ml of the buffer instead of enzyme solution. In each case the ratio of the enzyme to the substrate (16.6 U of collagenase per 1 mg of protein) was the same. Incubations were carried out at 37°C for 4 h. The reactions were stopped by the addition of EDTA-Na_2 to the final concentration of 5 mM. The effects of digestion were evaluated by the electrophoretic method and gel filtration as described below.

Sodium dodecyl sulphate/polyacrylamide gel electrophoresis. Electrophoretic mobility of AF proteins on slab polyacrylamide gels was examined by the method of Laemmli [18]. The samples of AF were combined with 1/4 their volume with 0.625 M Tris/HCl, pH 6.8, 15% SDS and 40% glycerol. The proteins present in solution were denatured by heating in boiling water for 5 min. Electrophoresis was carried out on 7.5%, 12% and 14% polyacrylamide gels containing 0.1% SDS. In each case a 6% stacking gel was applied. Electrophoresis was run at a constant current of 30 mA. The gels were stained with 0.125% Coomassie Brilliant Blue R-250. In some cases the silver-stain method was applied [19].

Gel filtration. Sephadex G-100 column (35.0 cm × 2.7 cm) was equilibrated with 50 mM ammonium hydrocarbonate and 2 ml samples were applied. Both dialyzed and non-dialyzed, 5-fold concentrated AF as well as the collagenase digestion products were submitted to this procedure. The material applied to the column was eluted with 50 mM NH_4HCO_3 solution and 4 ml fractions were collected. The content of proteins and peptides in eluates was estimated by absorbance at 280 nm and 230 nm. Fractions corresponding to individual absorption peaks were combined, lyophilized and dissolved in 2 ml of distilled water. Hyp and protein concentrations were determined in each of these pooled fractions.

The same method was applied for the assay of the molecular mass of AF constituents. The following standards were used: BSA (67 kDa), cytochrome *c* (13 kDa) and methylene blue (0.25 kDa, eluted in total volume of the column). Fractions of 4 ml were collected. The positions of standards in the eluate were localized by measuring the absorbance with the use of a Beckman DU 640 type spectrophotometer.

Collagen (type I) isolation. Frozen rat tail tendons were cut, suspended in 0.5 M acetic acid and extracted with continuous stirring for 18 h at 4°C. Insoluble material was separated by centrifugation (17000 × ***g***, 30 min) and discarded. Collagen present in the supernatant was precipitated with 1.7 M NaCl and centrifuged in the same conditions as previously. The precipitate (containing type I collagen) was dissolved in 0.5 M acetic acid and dialysed against the same solution (24 h, 4°C), and stored at 4°C.

RESULTS

Absorbance spectra of hydroxyproline chromogens

In the absorbance spectra of both the hydroxyproline chromogen developed for the standard of this imino acid and the corresponding chromogen of type I collagen hydrolysate an evident peak of absorbance, at 560 nm is visible (Fig. 1a). This spectrum is typical for chromogen formed in the reaction of pyrrole (product of carboxylation and oxidation of Hyp) with *p*-dimethylaminobenzaldehyde, as described by Woessner [16].

Non-dialyzed AF also gives a typical hydroxyproline colour reaction with the absorption spectrum (Fig. 1b) very similar to those observed in the cases of pure Hyp and pure collagen (Fig. 1a). However, after dialysis the peak at 560 nm, characteristic for Hyp, almost completely disappeared from the AF spectrum. The observed "flat" absorbance curve is not typical for hydroxyproline chromogen.

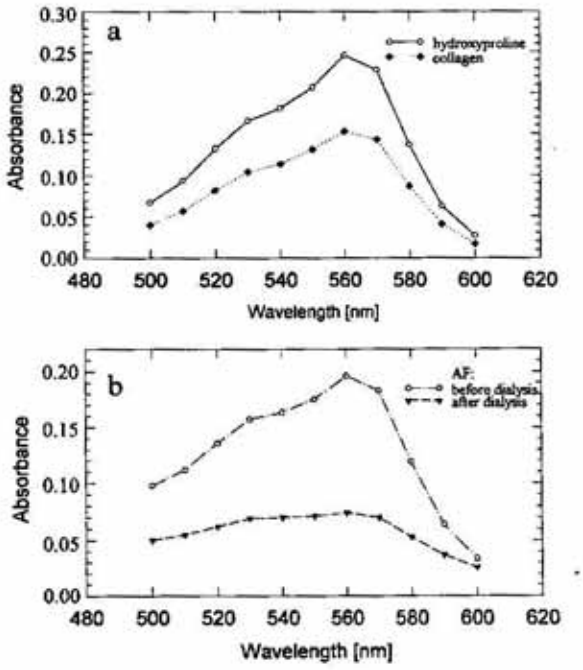

Figure 1. Absorbance spectra of hydroxyproline chromogen: Hyp and collagen solution (a) and amniotic fluid before and after dialysis (b).

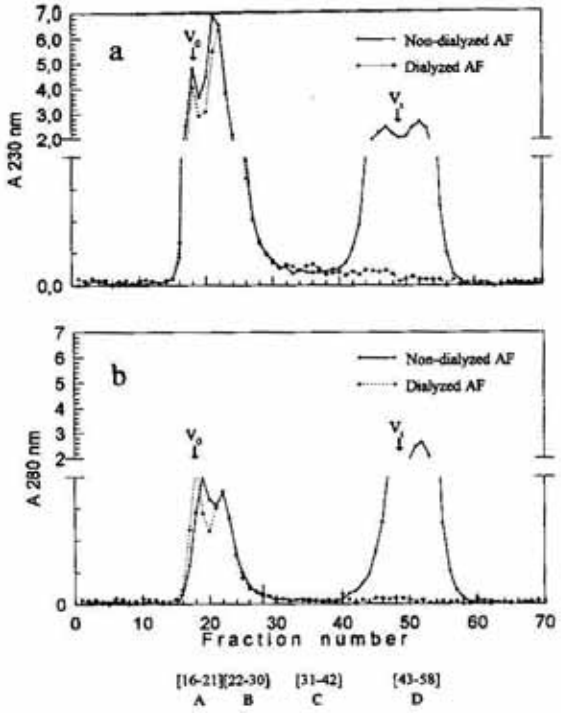

Figure 2. Sephadex G-100 chromatography of amniotic fluid.

Fractions of 4 ml were collected. Absorbance was measured at 230 nm (a) and 280 nm (b). The fractions pooled for further studies (A–D) are indicated.

Heterogeneity of hydroxyproline-containing constituents of AF

Gel filtration of non-dialyzed AF on Sephadex G-100 resulted in separation of its compounds into two peaks when the absorbance of fractions was measured at 230 nm and 280 nm. Dialysis did not affect the first peak but the second was reduced at both wavelengths (Fig. 2a and Fig. 2b).

The fractions of the eluate were pooled as indicated in Fig. 2, and four pooled fractions A, B, C and D were examined. They were concentrated by lyophilization and assayed for protein by the biuret method and Hyp content.

Fractions A and B contained high molecular mass products giving chromogens during the Hyp colour reaction. Similar results were obtained for both non-dialyzed and dialyzed material (not shown). It is worth of special note that the "interpeak" fraction C (which demonstrated very low UV-light absorbance, approx. 0.1 at 230 nm) contained a significant amount of the chromogen. Dialysis reduced by half quantity of chromogen in this fraction (Fig. 3a). The last fraction (D) contained the highest amount of chromogen, bound to low molecular, dialyzable material (Fig. 3a). In connection with low absorbance at 280 nm of dialyzed fractions C and D it should be remembered that this is due to a very low content of aromatic amino acids in collagen-derived compounds [1].

The quantitative ratios of Hyp-like reacting compounds to protein are very low in fractions A and B (not shown). It may be calculated that such compounds constitute approx. 0.12% of the mass of proteins present in these two fractions, and that dialysis did not affect this parameter. Since proteins in fractions A and B constituted 93.46% of total AF proteins, this would mean that Hyp in these fractions amounted 0.11% of total AF proteins, whereas in the fractions C and D – 0.08% of total AF proteins. In contrast to A and B frac-

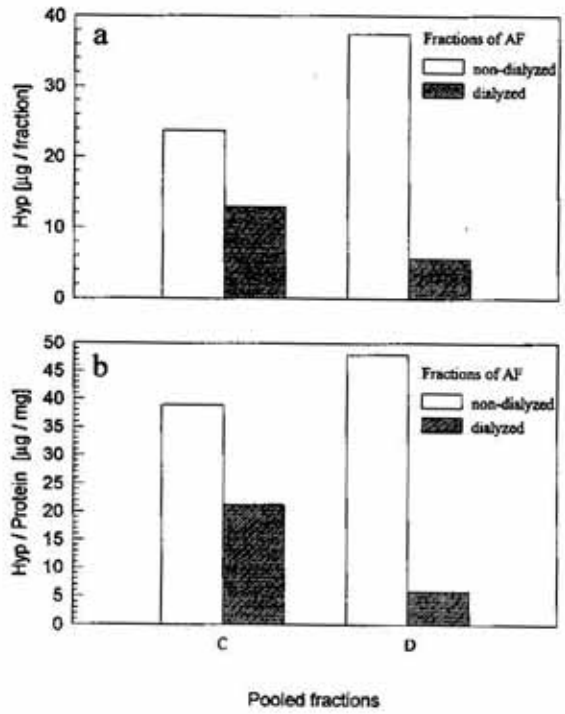

Figure 3. Hyp content in the fractions C and D as indicated in Fig. 2 (a) and the Hyp:protein ratio in these fractions (b).

tions, the Hyp:protein ratios in fractions C and D were by several-fold higher and they distinctly decreased in the dialyzed AF (Fig. 3b).

Differences in the hydroxyproline chromogen of AF fractions

Essential differences were noted between the absorption spectra of the chromogens arising during the Hyp colour reaction, developed for fractions A, B, C and D. The chromogens in the hydrolysates of fractions A and B did not show the spectra typical for hydroxyproline. No separate peak at 560 nm was observed (Fig. 4).

In contrast, fractions C and D, containing the products of lower molecular mass, display the absorption spectra characteristic for hydroxyproline (Fig. 4).

Lack of hydroxyproline-containing proteins in fractions A and B has been confirmed using bacterial collagenase.

Collagenase digestion of AF did not alter the amounts of Hyp-like reacting compounds either in fraction A or B. The ratios of Hyp-like reacting compounds to protein in these frac-

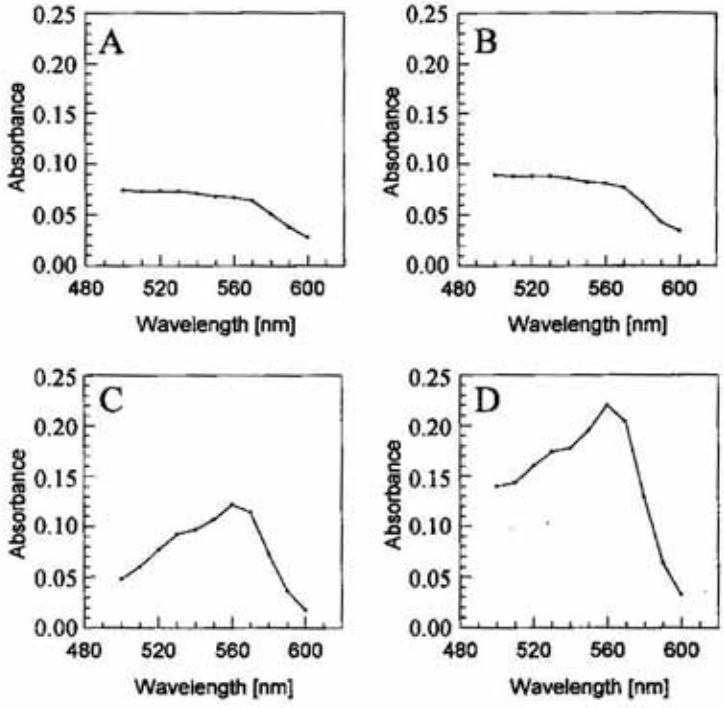

Figure 4. Absorbance spectra of hydroxyproline chromogen of the fractions: A, B, C and D of amniotic fluid (see Fig. 2).

tions did not change under the action of collagenase (not shown). In contrast, treatment with collagenase distinctly decreases hydroxyproline content in dialyzed fraction C (not shown). This point to the collagenous character of protein(s) present in the lower molecular fractions of AF.

Electrophoretic characterization of amniotic fluid proteins

Electrophoretic mobility of AF proteins on polyacrylamide gel in comparison to other proteins (BSA and collagen subunits) is presented in Fig 5a. Lane 2 shows that the predominant fraction of AF proteins migrated at a rate corresponding to that of BSA. Another protein band migrated close behind the main fraction. A broad protein band corresponding to high molecular mass proteins is apparent close to the top of the gel (Fig. 5a).

The electrophoretic mobilities of protein constituents of fractions A and B did not change on collagenase treatment (Fig. 5b), therefore they cannot contain collagenous products corresponding to α-subunits. In contrast, the composition of fraction C changed under the action of this enzyme (Fig. 5c). Three bands marked in this figure by asterisks disappeared after collagenase treatment.

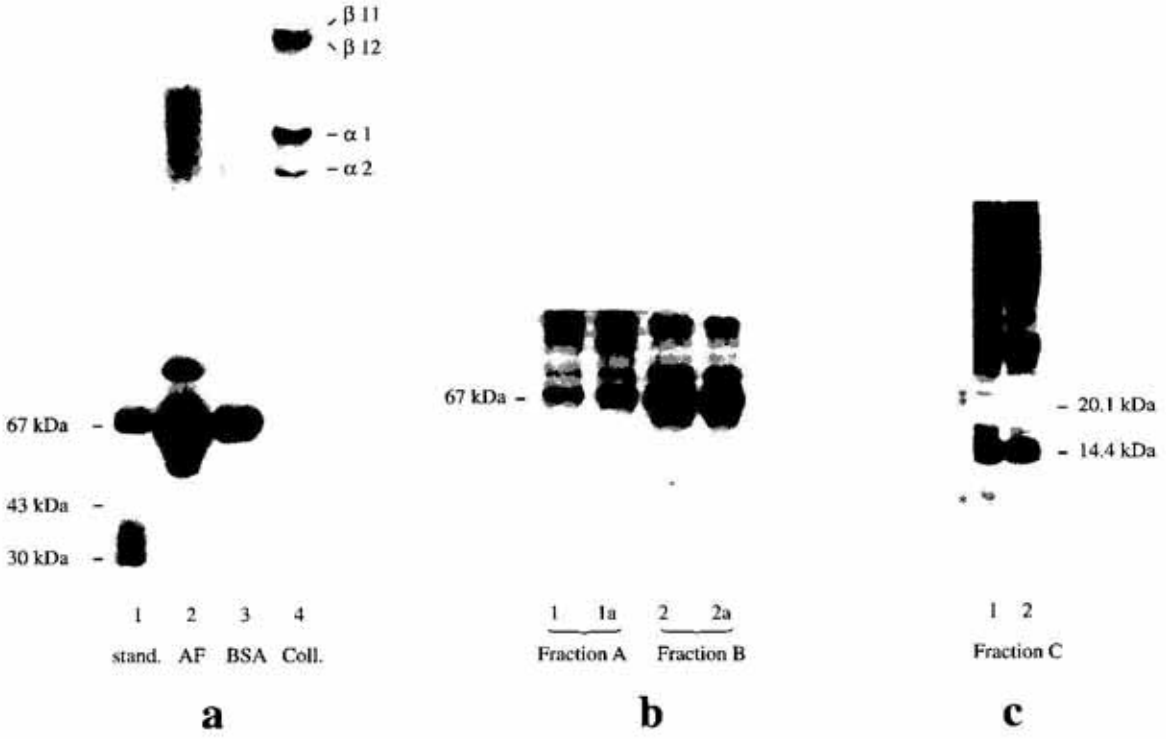

Figure 5. SDS/PAGE of amniotic fluid (a) and the fractions A and B (b) and fraction C (c). Electrophoresis was performed on 7.5% (a), 12% (b), and 14% (c) gels stained with Coomassie Brilliant Blue R-250 (a and b) or by the silver method (c).

a) Lane 1, molecular mass standards: bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa). Lane 2, AF proteins (dialyzed). Lane 3, bovine serum albumin (67 kDa). Lane 4, α_1 and α_2 subunits of the rat tail tendon collagen type I (about 100 kDa) and their dimers, β_{11} and β_{12} (about 200 kDa).

b) Lane 1 and 1a, fraction A. Lanes 2 and 2a, fraction B. Lanes 1 and 2, non-digested proteins. Lanes 1a and 2a, proteins treated with bacterial collagenase.

c) Lane 1, non-digested sample. Lane 2, sample digested by bacterial collagenase. Positions of soybean trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.4 kDa) are marked. Proteins disappearing after digestion by collagenase are marked by asterisks.

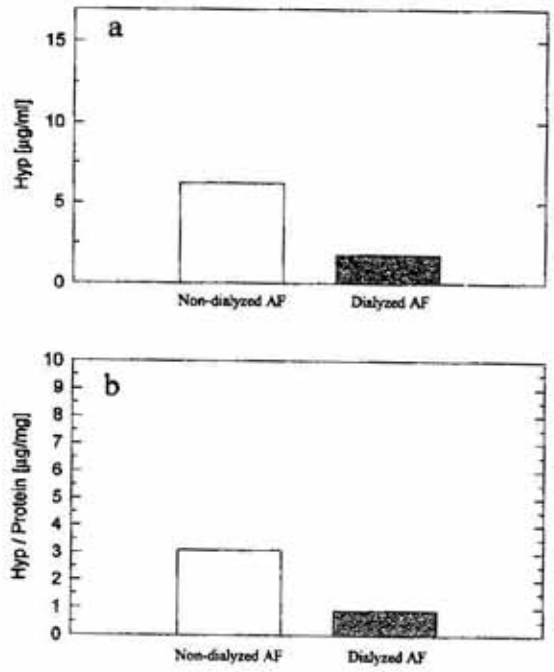

Figure 6. Hyp concentration (a) and Hyp:protein ratio in the amniotic fluid. The Hyp colour reaction interfering material (fractions A and B) was removed by the Sephadex G-100 gel filtration.

Two of them corresponded to proteins of about 20 kDa and the third one, of less than 14 kDa (Fig. 5c). This allows to conclude that only fraction C contained some (at least 3) collagenous polypeptides.

Estimation of molecular mass of proteins present in fraction C obtained after Sephadex G-100 column gel filtration indicated that the collagenous polypeptides are a mixture of proteins of about 7.9–26.3 kDa, while the molecular mass of the compounds in fraction D were below 8 kDa.

Protein and hydroxyproline contents in amniotic fluid

Gel filtration allows to separate the collagen degradation products from the other Hyp-like reacting compounds. Basing on separation of the non-specific Hyp chromogens it can be concluded that 1 ml of AF contains 6.2 μg of Hyp. Most of Hyp-containing products are low molecular dialyzable substances. Only 1.8 μg of Hyp in 1 ml of AF is bound to non-dialyzable collagenous products.

The mean protein content in 33 dialyzed samples of AF was 1.98 ± 0.46 mg/ml. Taking into account this result, the Hyp:protein ratio in the AF equals to 0.031 before dialysis and 0.009 after dialysis (Fig. 6).

DISCUSSION

It is thought that determination of Hyp concentration in amniotic fluid can serve as an index of fetal development [20]. Our primary studies demonstrated that non-fractionated AF contains Hyp in concentration of about 10 μg/ml, which might correspond to 80 μg of collagen or its degradation products. Similar values were reported by Shah *et al.* [20] as well as by Bissenden *et al.* [21].

However, we have demonstrated that these values are overestimated, probably due to the interference of some other constituents of this fluid in the Hyp colour reaction. Therefore it should be concluded that the method of Woessner, commonly used by researchers worldwide, is of limited value in the case of AF.

Hyp concentration has been recalculated, following gel filtration, to be of about 6.2 μg/ml. The Hyp concentration in dialyzed amniotic fluid decreased to about 1.8 μg/ml, which allows to conclude that 30% of hydroxyproline present in AF is bound to non-dialyzable products (Fig. 6), while the major part is contained in low molecular peptides, or it exists as a free Hyp.

Both the tissues of mother and fetus participate in formation of AF components. Fetal urine is the main source of various peptides that represent degradation products of fetal proteins, including collagen. Both free and short peptide-bound Hyp, reflecting the degradation process of mature collagen [22], may diffuse through amniotic membranes, so

their content in the AF may depend on maternal diet and metabolism.

That part of hydroxyproline which is bound to nondialyzable polypeptides of higher molecular mass, could serve as a more specific index of the metabolism rate of fetal collagen. One may assume that the Hyp-containing nondialyzable peptides, like most high molecular mass proteins, do not penetrate the fetal membranes, at least not to a high extent [20]. The Hyp-containing macromolecular compounds of AF may therefore be considered as the fetal metabolism products. In adult subjects about 10% of total urine Hyp is in the form of such collagenous polypeptides [23]. Their concentration in urine increases when intensive biosynthesis of collagen takes place, e.g. during the growth and maturation periods [20], Paget's disease of bone [23] or some neoplasmatic processes [24].

It is apparent from our study that the Hyp-containing products in AF are a mixture of peptides of various molecular mass. Approximately 70% of them are the low molecular, dialyzable peptides, represented in our material by fraction D. We have found that the amniotic fluid does not contain those high molecular mass forms of collagen, which correspond to α-subunits. The high molecular AF proteins (fractions A and B) did not form a typical chromogen in the colour reaction for hydroxyproline. Furthermore, they were resistant to the action of bacterial collagenase. Therefore they cannot be considered collagenous proteins.

Special attention should be paid to the "interpeak" fraction C. No doubt, it contains non-dialyzable products of collagen degradation. Such a conclusion results from our observations showing a significantly high Hyp content in this material, its susceptibility to the action of bacterial collagenase and formation of a typical chromogen in colour reaction for hydroxyproline. Molecular mass of these products, estimated by gel filtration and supported by SDS/PAGE, appears to be in the range of 7.9–26.3 kDa. They are most probably the polypeptide products of collagen degradation.

One may conclude that fetal urine is the main source of those Hyp-containing polypeptides that may be considered the fetal collagen degradation products. Krane *et al.* [23] described the presence of high molecular (4–10 kDa), Hyp-containing peptides in urine of adult individuals. These polypeptides are considered as degradation products of newly synthesized collagen. We think that such collagenous polypeptides may be the products of proteolytic conversion of the collagen precursor (procollagen) into monomeric form of this protein. These products might include N-terminal propeptides of α_1 chains of type I procollagen (PINP) [25] and those of type III procollagen (PIIINP) [26], as well as the products of their proteolysis. These propeptides contain short triple helical segments (about 50 amino-acid residues). Such triple helical domains are susceptible to the action of bacterial collagenase and resistant to the action of nonspecific proteolytic enzymes. Since AF does not contain collagenolytic activity (unpublished) these polypeptides cannot be cleaved there into low molecular products.

On the other hand, the presence of cross-linked polymeric collagen breakdown products in AF cannot be excluded. Such a possibility results from observations of Macek & Adam [27], who described the presence of Hyp-containing cross-linked peptides of molecular mass exceeding 10 kDa in urine of adult individuals.

The results presented in this paper indicate that amniotic fluid does not contain macromolecular forms of collagen but only its degradation products of molecular mass not higher than 26.3 kDa.

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