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Pregnancy-related changes in rat cervical glycosaminoglycans

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Non-pregnant and pregnant rats of known gestational age were killed at intervals and their uterine cervices were excised and digested with papain. Glycosaminoglycans thus extracted were separated by cellulose acetate electrophoresis and stained with Alcian Blue. Glycosaminoglycans were identified by comparison with standards and by serial degradation with chondroitin ABC lyase, butyl nitrite and leech hyaluronidase. Dermatan sulphate, hyaluronic acid and heparan sulphate were identified and quantitatively determined by densitometry. The overall concentration of glycosaminoglycans changed little during pregnancy. A 3-fold total increase in uronic acid paralleled the increase in cervical weight. Hyaluronate content, however, increased 17-fold, and rose from 6% of total glycosaminoglycans in the non-pregnant state to 33% at term. Furthermore, the ratio of hyaluronate to hydroxyproline increased 10-fold. These changes are consistent with an accumulation of hyaluronate in the interstices between collagen fibres, resulting in the softening of this tissue that is seen in late pregnancy.

While the uterine fundus enlarges during pregnancy, the uterine cervix remains firm and closed to contain the conceptus. Then, late in pregnancy and during labour, the fibrous connective tissue of the cervix softens to allow dilatation and easy egress. This physical change in the cervix is commonly thought to be a passive response to uterine contractions. More recent evidence suggests, however, that softening results instead from intrinsic alterations in the extracellular components of the stroma (Danforth *et al.*, 1960, 1974; Zarrow & Yochim, 1961; Buckingham *et al.*, 1962; Cretius *et al.*, 1966; Bryant *et al.*, 1968; Kondo, 1972; Danforth & Buckingham, 1973; Rimmer, 1973; von Maillot & Zimmermann, 1976; Kleissl *et al.*, 1978). Indeed, initiation of cervical softening and onset of uterine contractions may, at least in some species, be under independent hormonal control (Stys *et al.*, 1978).

Investigations of metabolic changes in cervical connective tissue that might produce these physical effects have dealt for the most part with alterations in the collagen composition. Only preliminary studies have been done on the mucopolysaccharide components of the matrix. Among these have been reports by Danforth *et al.* (1974) and Breen *et al.* (1975), who isolated a fraction containing glycosaminoglycans from non-pregnant and term-pregnant human cervical biopsies and determined its hexosamine and uronate content. They noted that the hexosamine concentration was lower and the hexosamine/uronate ratio higher in the term cervix compared with the non-pregnant cervix and interpreted these results as indicating a decrease in glycosaminoglycan concentration and a change in polysaccharide composition. von Maillot & Greiling (1977) extracted a glycosaminoglycan fraction from non-pregnant and term human cervices by papain hydrolysis and partially characterized it by Dowex chromatography. They reported a 2.5-fold increase in fractions identified as 'hyaluronate' and 'keratan sulphate' and a 5-fold increase in 'chondroitin', whereas the concentration of 'chondroitin sulphate plus dermatan sulphate' changed little.

Bryant *et al.* (1968) hydrolysed rat cervices with HCl and measured hexosamine and uronate contents throughout pregnancy. They reported a 5-fold increase in hexosamine concentration and a 10-fold increase in total hexosamine in term compared with non-pregnant cervix (stroma plus endocervix). Moreover, most of this increase occurred just before parturition. They did not isolate macromolecular components, however.

These studies indicate that marked changes in cervical stromal mucopolysaccharide may occur during pregnancy and parturition. It is possible that alterations in the composition of the polysaccharide element of the matrix may affect interactions between collagen fibres and thus change the physical properties of the tissue. For these reasons

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we undertook a characterization of the glycosaminoglycan composition of the rat cervix during pregnancy.

Experimental

Materials

Papain (type II) and 9-aminoacridine hydrochloride were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Butyl nitrite, Alcian Blue and *m*-phenylphenol were obtained from Eastman Kodak Co., Rochester, NY, U.S.A. Hyaluronic acid, chondroitin sulphate A, chondroitin sulphate C, dermatan sulphate, chondroitin ABC lyase (EC 4.2.2.4) and chondroitin AC lyase (EC 4.2.2.5) were obtained from Miles Research Products, Chicago, IL, U.S.A. Leech hyaluronidase (hyaluronate 3-glycanohydrolase, EC 3.2.1.36) was obtained from Biotrics, Riverdale, NY, U.S.A. Spectrapor dialysis tubing was obtained from Spectrum Medical Industries, Queens, NY, U.S.A., and Sepraphore III cellulose acetate sheets and Sepraclear solution from Gelman Instrument Co., Ann Arbor, MI, U.S.A. Dowex AG 50W was obtained from Bio-Rad Laboratories, Richmond, CA, U.S.A. All other chemicals were of the highest purity commercially available.

Tissues

Sprague–Dawley rats of known gestational age and non-pregnant rats of the same age were obtained from Charles River, Wilmington, MA, U.S.A. Rats were killed by an overdose of chloroform, and the lower uterine horns, cervix and upper vagina were excised *en bloc* and placed in chilled 0.9% NaCl. The vagina was then trimmed from the cervix and the uterine horns were removed at their bifurcation. Cervices were opened longitudinally, rinsed in 0.9% NaCl to remove mucus, blotted and weighed.

Isolation of glycosaminoglycans

Cervices were individually dry-defatted by shaking at 4°C in chloroform/methanol (2:1, v/v) for 24h followed by extraction with acetone for 12h. Tissue was then dried under a stream of air, weighed and suspended in 1.0ml of buffer A (0.1M-KH_2PO_4/10mM-EDTA/10mM-cysteine hydrochloride, adjusted to pH6.7 with KOH)/50mg original wet weight. Papain, purified from Sigma type II preparation by the method of Kimmel & Smith (1954), was added to a final concentration of 0.5mg/ml and the suspension was heated at 65°C for 72h (Rodén *et al.*, 1972). After samples had been withdrawn for hydroxyproline determination, 100% (w/v) trichloroacetic acid solution was added to give a final concentration of 5% (w/v). After the mixture had stood overnight, the precipitate was removed by centrifugation. The pellet was washed twice with 1.0ml of 5% trichloroacetic acid and the supernatants were combined.

The supernatant was dialysed against three changes of 20vol. of water, Spectrapor 1 membrane tubing (6000–8000-mol.wt. exclusion limit) being used. The dialysis residue was freeze-dried, redissolved in a minimal amount of water and precipitated by the dropwise addition of an aqueous solution of 9-aminoacridine hydrochloride (saturated at 60°C) until no further turbidity was produced (Tsiganos & Muir, 1969). The glycosaminoglycan–aminoacridine complex was left overnight and collected by centrifugation. The complex was dissolved by the addition of Dowex AG 50W (X16; Na^+ form) and the resin suspension was filtered on a glass funnel and washed with 3vol. of water. A sample of the combined filtrates was withdrawn for uronate determination.

Identification and quantitative determination of individual glycosaminoglycans

Cellulose acetate electrophoresis was carried out on Sepraphore III membranes by using a Gelman Sepratek micro-electrophoresis chamber and applicator. Solutions to be electrophoresed contained approx. 1.0mg of uronate/ml for each glycosaminoglycan. Electrophoresis in cadmium acetate buffer (0.3M-cadmium acetate adjusted to pH4.1 with acetic acid) for 1h at 4.4mA was used to separate hyaluronic acid, heparan sulphate, dermatan sulphate and chondroitin sulphates (Curwen & Smith, 1977). Electrophoresis in calcium acetate buffer (0.2M-calcium acetate adjusted to pH7.0 with acetic acid) for 5h at 5.0mA was used to separate chondroitin 6-sulphate, chondroitin 4-sulphate and dermatan sulphate from a mixture of heparan sulphate and hyaluronic acid (Stanbury & Embery, 1977).

Electrophoresis membranes were stained with 1% (w/v) Alcian Blue in ethanol/50mM-sodium acetate (1:1, v/v) for 8min and then destained with acetic acid/ethanol/water (1:2:17, by vol.) (Breen *et al.*, 1976). The membranes were treated with Sepraclear and mounted on glass slides. Densitometry was performed with an Autoscanner desitometer with a 610nm filter and a Quick Quant II digital computer (Helena Laboratories, Beaumont, TX, U.S.A.).

The concentrations of standard glycosaminoglycan solutions were determined by hydrolysis in 8M-HCl at 95°C for 3h and hexosamine analysis by ion-exchange chromatography. Integration of the electrophoretogram in the direction of migration allowed the determination of the absorptivities of Alcian Blue adducts of standard glycosaminoglycan solutions. The plot of absorbance versus glycosaminoglycan concentration was linear from 0.2 to 2.0mg of uronate/ml. Relative concentrations of glycosaminoglycans in each preparation of cervix

were determined by integration in the direction of electrophoretic migration. Cervical glycosaminoglycans were assumed to have absorptivities identical with those of co-migrating standards. Absolute concentrations were determined by turning the plate through 90° and integrating a series of well-resolved co-migrating bands. The standard deviation of four hyaluronate standards of identical concentration treated in this manner was 6% of the mean value. In the usual case, electrophoresis was performed in cadmium acetate buffer and the hyaluronate content of each sample was related to the hyaluronic acid standard. Integration of each sample run in cadmium acetate buffer in the direction of migration was also used to determine the concentration of the sum of fast- and slow-moving dermatan sulphate. The relative proportions of these latter glycosaminoglycans were determined by electrophoresis in calcium acetate buffer.

Confirmation of glycosaminoglycan identity

(*a*) *Chondroitin sulphate lyase digestion.* Two samples of a glycosaminoglycan mixture from pooled term cervices, each containing 50 μg of uronate, were evaporated and resuspended in 100 μl of a buffer solution of 0.1 M-sodium acetate and 0.1 M-Tris base adjusted to pH 8 with HCl. Chondroitin ABC lyase (0.1 unit) or chondroitin AC lyase (0.3 unit) was added, and digestion was carried out at 37°C for 1 h (Saito *et al.*, 1968). The reaction was stopped by heating the solutions at 100°C for 5 min. After repeated dialysis against water, the solutions were dried under a stream of air, reconstituted in 20 μl of water and spotted (0.25 μl) on cellulose acetate for electrophoresis.

(*b*) *Butyl nitrite degradation.* The method of Cifonelli & King (1972) was used. Half of the solution of glycosaminoglycans remaining after chondroitin ABC lyase degradation was taken up in water and put through a 0.2 ml column of Dowex AG 50W (X16; H^+ form). The column was washed with 0.5 ml of water, and combined eluates were dried under a stream of air and reconstituted in 50 μl of water. A 0.1 ml portion of a solution of 0.5 ml of butyl nitrite in 12.5 ml of 1,2-dimethoxyethane at −20°C was added. The solution was mixed well and kept at −20°C for 4 h. It was then dried under a stream of air, reconstituted with water and subjected to electrophoresis.

(*c*) *Leech hyaluronidase digestion.* Half of the glycosaminoglycan mixture remaining after chondroitin ABC lyase digestion was taken up in 100 μl of 0.12 M-Na_2HPO_4/42 mM-citric acid, pH 5.6, and 10 μg of leech hyaluronidase was added. The reaction was carried out at 37°C for 24 h. Another 10 μg of hyaluronidase was added after 8 h. The reaction was stopped by heating at 100°C for 5 min. The solution was dialysed against water, evaporated and electrophoresed.

Analytical

Hexuronic acid was determined by the method of Blumenkrantz & Asboe-Hansen (1973). Interference by non-specific chromogen formation was encountered with all H_2SO_4 methods and necessitated hydrolysis and removal of protein in all cases to assure accurate results. Glucosamine and galactosamine were determined by ion-exchange chromatography on a Beckman 119 C amino-acid analyser of samples hydrolysed at 95°C in 8 M-HCl for 3 h. Hydroxyproline was determined by the method of Woessner (1961).

Results

Fig. 1 shows the variation of total cervical uronate content throughout pregnancy and the puerperium. Uronate content changed little over the first 2 weeks of gestation but then rapidly increased 3-fold by term. In the post-delivery period there was a rapid

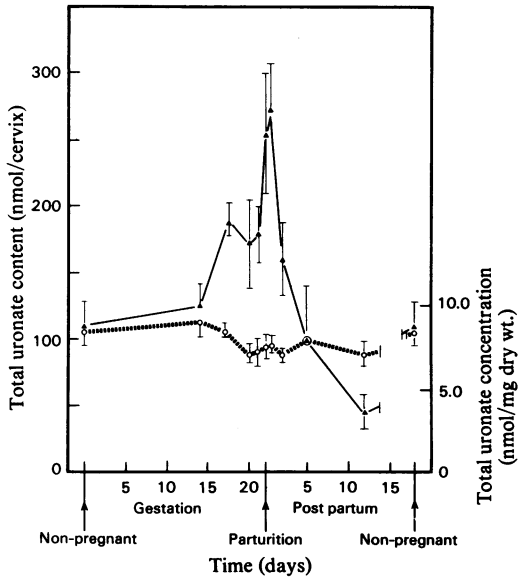

Fig. 1. *Hexuronic acid content* (▲) *and concentration* (○) *in rat uterine cervix throughout pregnancy*
Cervices were excised from pregnant and non-pregnant rats at the indicated times. Glycosaminoglycans were extracted by papain hydrolysis and purified by precipitation with 9-aminoacridine, and the uronate content was determined as described in the Experimental section. Each point represents the mean of values obtained from five individual cervices, except for the 'Parturition' value, which represents the mean of values from nine cervices. Vertical bars represent ± 1 S.D.

decline to values lower than that in the pre-pregnant state. These changes, however, closely paralleled the change in cervical wet weight from a non-pregnant-state value of 77 ± 13 mg (mean $\pm$ S.D.) to 225 ± 25 mg at term. Since the water content changed only from 83% in the non-pregnant state to 85% at term, the concentration of total uronate remained nearly constant at 7–9 nmol/mg dry wt.

Hydroxyproline content also changed little over the first 2 weeks of pregnancy, but then increased 2-fold over the last week (Fig. 2). Since the cervical weight increased 3-fold, however, the concentration of hydroxyproline at term fell to two-thirds of the non-pregnant-state value and the ratio of uronate to hydroxyproline increased by 50% by term.

Individual glycosaminoglycan concentrations were determined by cellulose acetate electrophoresis after proteolysis with papain. Since it was not possible to determine the glycosaminoglycan composition of individual cervices accurately, groups of five cervices were used.

With the use of cadmium acetate buffer at pH 4.1, three glycosaminoglycans were identified. The major glycosaminoglycan was found to have a mobility similar to that of standard pig skin dermatan sulphate. Its identity was confirmed by its sensitivity to digestion by chondroitin sulphate ABC lyase and resistance to chondroitin sulphate AC lyase.

A glycosaminoglycan co-migrating with standard hyaluronic acid was identified by electrophoresis in cadmium acetate buffer. Its identity was confirmed by its resistance to digestion by chondroitin sulphate ABC lyase and butyl nitrite and its sensitivity to degradation by leech hyaluronidase. A band migrating between hyaluronate and dermatan sulphate in cadmium acetate buffer was tentatively identified as heparan sulphate by its characteristic migration rate (Curwen & Smith, 1977) and by its resistance to digestion by chondroitin sulphate ABC lyase and leech hyaluronidase and sensitivity to butyl nitrite degradation.

The cervical content of dermatan sulphate changed little over the first 2 weeks of pregnancy (Fig. 3) and then increased to twice the non-pregnant-state value by term. The concentration of dermatan sulphate per unit dry weight (Fig. 4) or per μmol of hydroxyproline (Fig. 5) varied little

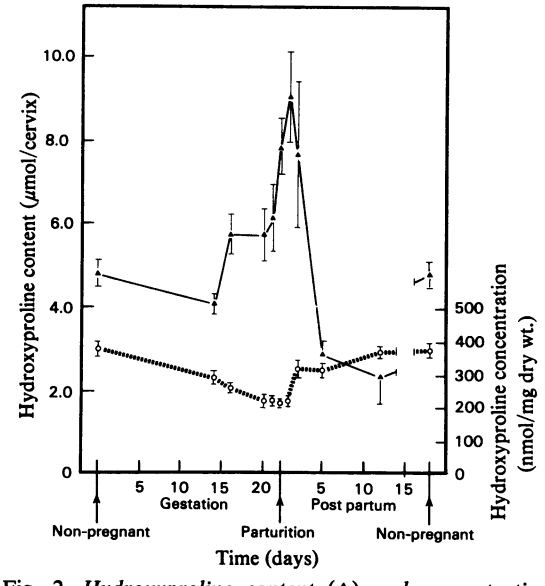

Fig. 2. *Hydroxyproline content* (▲) *and concentration* (○) *in rat uterine cervix during pregnancy*
Cervices were excised from pregnant and non-pregnant rats at the indicated times, hydrolysed with papain and hydroxyproline determinations were performed as described in the Experimental section. Each point represents the mean of values obtained from five individual cervices, except for the 'Parturition' value, which represents the mean of values from nine cervices. Vertical bars represent ± 1 S.D.

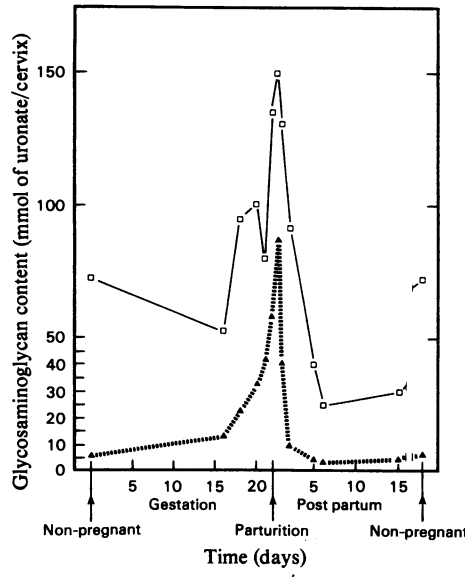

Fig. 3. *Dermatan sulphate* (○) *and hyaluronic acid* (▲) *contents per cervix throughout pregnancy*
Glycosaminoglycan mixtures were extracted from cervices excised in groups of five at the indicated times and assayed quantitatively by electrophoresis on cellulose acetate in cadmium acetate buffer as described in the Experimental section. Each point represents the total amount of each glycosaminoglycan in the pool of five cervices divided by the number of cervices.

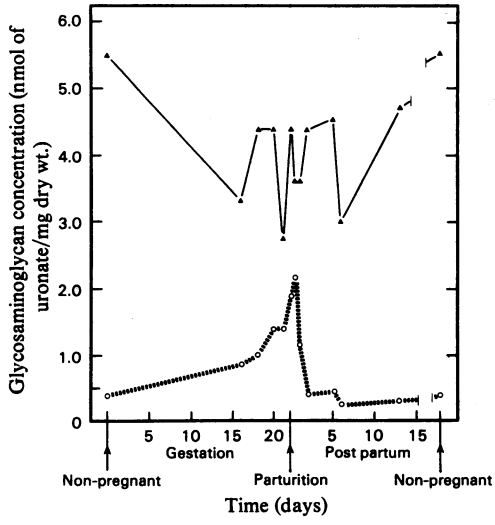

Fig. 4. *Concentration of dermatan sulphate* (▲) *and hyaluronic acid* (O) *per mg dry wt. of cervix throughout pregnancy*

Each point represents the mean amount of dermatan sulphate or hyaluronic acid determined for each group of five cervices excised at the indicated times as shown in Fig. 4 divided by the mean dry weight for that group of cervices.

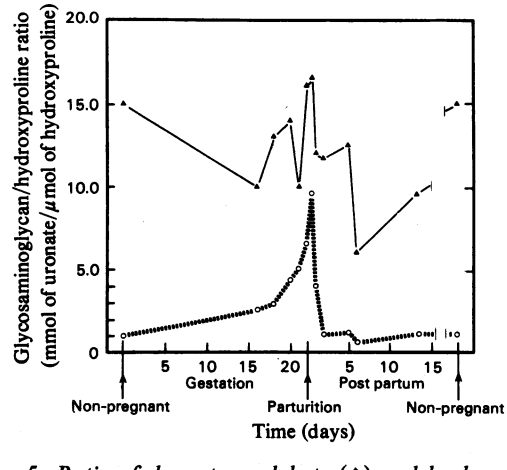

Fig. 5. *Ratio of dermatan sulphate* (▲) *and hyaluronic acid* (O) *contents to hydroxyproline content throughout pregnancy*

Each point represents the ratio of the mean amount of dermatan sulphate or hyaluronic acid per cervix determined as shown in Fig. 4 and the mean amount of hydroxyproline per cervix for groups of five cervices excised at the indicated times.

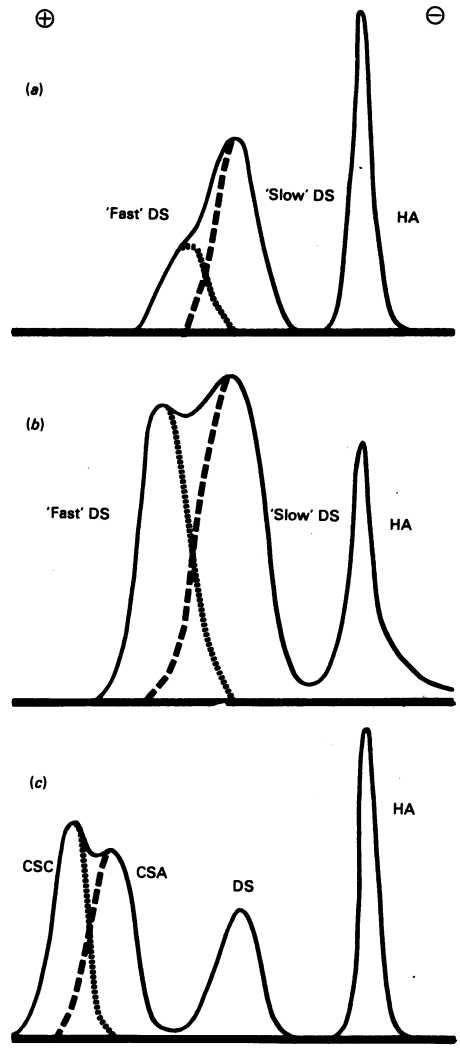

Fig. 6. *Densitometric tracings of calcium acetate electrophoretograms of glycosaminoglycans from pregnant and non-pregnant cervix*

Electrophoresis was performed in 0.2 M-calcium acetate, pH 7.0. Membranes were stained in Alcian Blue and subjected to densitometry as described in the Experimental section. Peak areas in this representation show only relative concentrations. The continuous line represents the actual tracing. Broken lines are derived component peaks assuming that they are symmetrical about the maximum value. In this buffer heparin sulphate migrates at that same rate as hyaluronic acid. (*a*) Glycosaminoglycans extracted from term pregnant-rat cervix. Abbreviations used: 'Fast' DS, rapidly migrating dermatan sulphate; 'Slow' DS, dermatan sulphate co-migrating with pig skin dermatan sulphate; HA, hyaluronic acid. (*b*) Glycosaminoglycans extracted from non-pregnant-rat cervix. (*c*) Standard glycosaminoglycans. Abbreviations used: CSC, chondroitin 6-sulphate; CSA, chondroitin 4-sulphate; DS, pig skin dermatan sulphate.

throughout pregnancy, however. Dermatan sulphate content fell precipitously post partum to one-third of the pre-pregnant-state value by 5 days after delivery. Thereafter, there was a gradual return to the non-pregnant-state content.

The dermatan sulphate band was partially resolved into two fractions by electrophoresis in calcium acetate buffer. One band co-migrated with pig skin dermatan sulphate and another migrated between dermatan sulphate and chondroitin 4-sulphate (Fig. 6). Assuming that each glycosaminoglycan band had a symmetrical distribution, component peaks were derived as indicated in Fig. 6 and integrated with a planimeter. The faster-moving component comprised about 43% of the total dermatan sulphate in the non-pregnant cervix, but only about 26% of that in term tissue.

Accurate estimation of 'heparan sulphate' content was not possible. Assuming an absorptivity for this substance similar to that of dermatan sulphate, however, 'heparan sulphate' decreased from a non-pregnant-state value of 15 nmol per cervix to 5 nmol per cervix at day 16, thereafter rising to 25 nmol per cervix at term.

Changes in hyaluronic acid content were much more marked. Hyaluronate increased more than 17-fold from 5 nmol of glucuronate per cervix in the non-pregnant state to 88 nmol of glucuronate per cervix immediately after delivery. Only 15% of this increase occurred before day 16 of gestation, whereas 50% occurred after day 21. The concentration per unit weight of hyaluronate (Fig. 4) increased 4-fold by term, and the content per μmol of hydroxyproline (Fig. 5) increased almost 10-fold, with most of that increase occurring after 20 days' gestation.

In the period post-partum there was a rapid decline in both the cervical content of hyaluronate and in its concentration until 5–6 days post partum the content was only half the non-pregnant value.

Discussion

Despite the wide variation in the morphology of the uterine cervix among mammals, in each species that has been studied the bulk of this organ has been found to be fibrous connective tissue. Consequently the structure and function of this organ is determined by the structure of the connective tissue.

During late pregnancy the human cervix 'ripens' or softens, and during labour it changes from a firm cylindrical structure with a length of 2 cm and a radius of 1–2 cm to a soft membranous structure 2–5 mm thick. At the same time the cervical canal dilates from a diameter of 2–3 mm to more than 10 cm, usually without tearing. Such a marked change, which could not occur in the non-pregnant state, must be possible because of marked alterations in the structure of the fibrous connective tissue during pregnancy. Qualitatively similar physical changes occur in the rat (Harkness & Harkness, 1959, 1961; Harkness & Nightingale, 1962), and thus are the basis for employing the rat cervix as a model of human cervical function.

It has been generally believed that, because of the rapidity of its occurrence, cervical ripening must result from depolymerization of the fibrous elements of the extracellular matrix. The results given in the present paper indicate rather that cervical softening parallels synthetic processes in the cervix, and suggest that the altered physical properties of this tissue are due instead to changes in the kind and proportions of fibrous and amorphous constituents.

As others have found (Harkness & Harkness, 1954, 1959, 1961; Zarrow & Yochim, 1961; Bryant *et al.*, 1968; Rimmer, 1973), total cervical collagen content in the rat as estimated from total hydroxyproline content increases steadily over the last week of pregnancy. During this time the cervix is becoming more dilated and distensible (Harkness & Harkness, 1959, 1961; Harkness & Nightingale, 1962). These findings indicate the formation of new collagen and are thus not compatible with degradation of pre-existing collagen as the sole explanation of cervical softening. This newly synthesized material could, however, interact differently with itself and with polysaccharide matrix elements to result in a more pliable tissue.

By the use of electrophoresis on cellulose acetate in two buffer systems and specific enzymic and chemical degradation, dermatan sulphate, hyaluronate and heparan sulphate have been identified as the main glycosaminoglycan constituents of cervical connective tissue.

The major glycosaminoglycan present in the cervix is dermatan sulphate. Its concentration was found to be about 4.5 nmol/mg dry wt., a value very similar to the concentration of galactosamine (3.5 nmol/mg dry wt.) reported by Damle *et al.* (1979) for a glycosaminoglycan preparation from pig dermis. During pregnancy there was a 2-fold increase in dermatan sulphate. Its concentration per unit weight or per μmol of hydroxyproline, however, varied only slightly. On the other hand, these results suggest (Fig. 6) that more than one type of dermatan sulphate may be present and that the proportions may change in pregnancy. Confirmation must await investigations of molecular size and microheterogeneity of dermatan sulphate and dermatan sulphate proteoglycan extracted from non-pregnant and pregnant tissue.

Dermatan sulphate has been shown to bind tightly to collagen (Öbrink, 1973; Öbrink & Sundelof, 1973; Öbrink *et al.*, 1975) and to direct the extra-

cellular formation of collagen fibrils (Toole & Lowther, 1968; Toole, 1976). In addition, it is the predominant glycosaminoglycan of proteoglycans found in tissues required to resist tension (Gillard *et al.*, 1977*a*,*b*). Changes in microheterogeneity during pregnancy, which were reflected in different charge density and thus different mobility on cellulose acetate, may well be the basis for the increased deformability of this tissue.

Much more striking than the metabolism of dermatan sulphate is the marked increase in hyaluronic acid content and concentration. Hyaluronate increased from 6% of the total glycosaminoglycans in the non-pregnant state to 33% at term, and the ratio of hyaluronate to hydroxyproline increased even more markedly. These findings indicate that hyaluronate accumulation in the cervix accelerates at the end of pregnancy to as much as 50nmol/mg dry wt. per 24h, or 3nmol/g wet wt. per h. This rate of accumulation is approx. 10-fold the rate of synthesis reported to occur in a hybrid mammalian cell culture line (Tomida *et al.*, 1974), but 80-fold less than that reported in Wilm tumour (Hopwood & Dorfman, 1978) and 50-fold less than that noted in rat fibrosarcoma (Hopwood *et al.*, 1974). Since degradation of hyaluronate occurs rapidly post partum, however, some degradation may already be occurring before delivery and thus the actual rate of synthesis may be even greater.

This change in the concentration ratio of hyaluronic acid to both dermatan sulphate and collagen is highly significant. Hyaluronic acid is a high-molecular-weight highly hydrated polymer of lower charge density than dermatan sulphate. Accumulation of this material in the interstices between collagen fibrils would disperse the fibrils and increase the distensibility of the tissue. Such a mechanism has previously been proposed on the basis of the increase in the hexosamine/hydroxyproline ratio in the rat cervix late in pregnancy (Harkness, 1956). More recently, Flint (1972) reported histochemical evidence of a similar mechanism of collagen dispersal by non-sulphated mucopolysaccharide in tissue remodelling after tendon transection.

Additional support for such a mechanism comes from studies by Orkin & Toole (1978) of hyaluronate content and hyaluronidase activity in developing chick-embryo heart. These authors found that the highest concentrations of hyaluronate were present early in development, when cell movement and tissue remodelling were the greatest, and that differentiation paralleled degradation of hyaluronate by endogenous hyaluronidase and the formation of dense connective tissue.

The results of the present study thus suggest that cervical softening results not from depolymerization of fibrous components, but from their dispersal by newly synthesized hyaluronic acid.

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