THE CHANGES IN CHEMICAL COMPOSITION DURING DEVELOPMENT OF THE BOVINE NUCHAL LIGAMENT

E. G. CLEARY, L. B. SANDBERG, and D. S. JACKSON

From the Department of Experimental Biology, University of Oregon Medical School, Portland, Oregon. Dr. Cleary's present address is Department of Experimental Pathology, Australian National University, Canberra, Australia. Dr. Sandberg's present address is Division of Biology, California Institute of Technology, Pasadena, California. Dr. Jackson's present address is Department of Medical Biochemistry, The University of Manchester, England

ABSTRACT

Whole bovine nuchal ligaments, or portions thereof (in the case of commercially valuable animals), were obtained from 45 animals (28 fetal and 17 postnatal) ranging in age from 110 days of gestation to 10 yr. Insoluble elastin was quantitatively prepared from the fresh ligaments by extraction with hot alkali and by a combination of multiple extractions with alkaline buffer and then repeated autoclaving. When adult samples were examined, the yields of insoluble residue by these two methods were very similar, but with young fetal samples the second method gave significantly higher values, because of incomplete purification of the elastin residue. The changes in the concentration of collagen, alkali-insoluble elastin, and DNA have been examined. DNA concentration, and, thus, cell population density, fell progressively during the fetal period of development, to reach a steady value soon after birth. Collagen appeared in appreciable quantities before elastin, but its concentration was rapidly halved at about the time of birth. Insoluble elastin concentration was low until the end of the 7th fetal month, at which time it began to rise rapidly. The rate of increase in elastin concentration remained high throughout the next 10–12 wk, by which time the adult value had been reached. Quantitative studies, on the basis of the whole ligament, showed that the total cell content rises to a maximum at birth, but falls soon after to a level about half that at birth. Total collagen production and elastin deposition continue at a steady, maximal rate over the interval from 235 days of gestation to the end of the 1st postnatal month. It is concluded that the immediate postnatal period would be the most favorable phase in which to attempt the isolation of the soluble precursor elastin.

The adult nuchal ligament of the larger mammals has the highest content of elastin of any tissue. Elastin can be isolated from this tissue, is relatively pure form and in high yield, by comparatively mild methods (1). This has been presumed to be associated with the relatively simple structure of the tissue, a fact confirmed by the many morphological studies to which it has been subjected (2–8). The developing bovine ligament has been the subject of only one previous chemical study, by Mall in 1896 (9).

Our own interest in this tissue stems from its potential value as a source of tropoelastin, the presumed soluble precursor of the insoluble elastin fibers. Initially, it was assumed that the rate of ligament growth, and so by inference, the proportion of extractable soluble elastin, would parallel the rate of deposition of mesodermal protein in the

developing bovine, and so would be maximal in the 5th postnatal month (10). However, it was soon apparent from studies on swelling and protein extractability (11) that the bovine ligament at this age behaved very much as did the adult material.

Accordingly, a study was set up to examine the changes in the chemical composition of the bovine ligament during its development, with the major emphasis on the changes in elastin contents. Concurrently, histological studies were made on sections taken from these same ligaments. The findings from this work are reported in the accompanying paper (12).

As whole ligaments were available for study in the fetal and early postnatal animals, it was possible to extend the findings to include quantitation of the absolute amounts of the major structural components, and so to determine their relative rates of growth throughout development. From this, it has been possible to identify that phase in the development of the ligament when elastin deposition is maximal and so, by immediate inference, the most profitable stage at which to seek to isolate the tropoelastin.

EXPERIMENTAL

Materials

Whole ligaments, or portions thereof, were obtained from a selection of bovines ranging in estimated age from 100 days of gestation to 10 yr. The majority of the fetal samples were taken from Hereford or Jersey cows; the early postnatal samples (up to 2 months of age) were taken from either Jersey or cross-bred calves of various origins; and the older specimens were obtained from a variety of different breeds, as opportunity presented for obtaining ligaments of known ages. Samples were secured from 45 animals—28 fetal and 17 postnatal.

The ligaments were obtained fresh from the slaughterhouse, within 15 min of the death of the animal, or, in the case of the fetal calves, within 20 min of the death of the mother. The ages of the fetal calves were estimated by comparison of their forehead-rump lengths with those detailed by Bogart (13). For the majority of the postnatal animals, the age was known exactly from the recorded date of birth. In those few instances (adult animals ages 1–3 yr) in which this information was not available, the age of the animal was estimated by the Veterinary Officer in attendance, from an examination of the dentition.[1]

[1] The authors acknowledge the assistance and cooperation of the staff of Armour and Co., Portland, Oregon.

Where practicable (up to the age of 53 days), the whole of the nuchal ligament was removed intact, and it was then cleaned of adherent fat and connective tissue, including the surrounding fascia. The ligament was then weighed, and representative sections were taken for histological examination. The remainder of the tissues was sealed in an air-tight container and frozen until ready for use—usually within 6–24 hr. Some tissues were stored thus at −20°C, and they have been used for further analyses up to 12 months after collection. Collagen and elastin determinations on these stored tissues were not significantly affected by this treatment.

Methods

The fresh or freshly thawed ligament was cut into pieces, which were crushed to a fine powder, using liquid nitrogen, in a stainless steel mortar. From this frozen, powdered mixture, duplicate samples were taken for the chemical analyses, or for prior exhaustive extraction with aqueous buffers.

CHEMICAL ANALYSES: *Water* content was determined gravimetrically, after drying to constant weight in an oven at 110°C. *Nitrogen* was determined by the micro-Kjeldahl procedure (14).

DEOXYRIBONUCLEIC ACID (DNA) DETERMINATION: Duplicate samples were further fragmented to a fine suspension in ethanol with a Virtis (The Virtis Co. Inc., Gardiner, N.Y.) microhomogenizer. After extraction of the lipids and acid-soluble materials (15), DNA was extracted by heating at 90°C in 5% trichloroacetic acid (TCA) for 15 min. In several instances, the washed samples were reheated in a further portion of 5% TCA for an additional 15 min, but the increase in DNA yield was insignificant (16). DNA content was determined in duplicate, by the diphenylamine method (17), with the exception that the light absorption was measured at two wavelengths (610 and 650 mμ) and the difference used for the calculation of the DNA content (18). Highly purified and highly polymerized DNA from calf thymus (Sigma Chemical Co., St. Louis) was used for the preparation of standard solutions.

DETERMINATION OF THE ALKALI-INSOLUBLE ELASTIN: Duplicate samples of the crushed fresh ligament were suspended in 0.1 N NaOH (10 volumes) and heated at 98°C for 50 min (after Lansing et al., 2). The residue was washed three times with cold 0.1 N NaOH and then with distilled water until the supernatant was neutral. The residual elastin was then dehydrated with ethanol and ethanol:ether and extracted overnight with ether. The final residue, after preliminary drying in air, was dried in an oven at 110°C to constant weight. Duplicate analyses of the elastin yield using this procedure agreed within ±1%.

EXTRACTION PROCEDURE: Samples (2–50 g) of crushed ligament were taken for the preparation

of insoluble elastin by a combination of exhaustive extraction with aqueous buffers followed by repeated autoclaving (modified from Partridge, Davis, and Adair, 1).

Each sample was converted to a very fine suspension in 10 volumes of 0.15 M Tris-saline buffer, by repeated short treatments in a Virtis homogenizer in the cold (0–5°C). The buffer was made by adding sodium chloride to an 0.02 M Tris solution until the final molarity was 0.15. The pH was adjusted to 8.4 by the addition of hydrochloric acid. Under these conditions, the swelling of the ligaments and the extraction of protein are maximal (9). A few drops of sec-octanol were added to the buffer for each extraction, both to reduce foaming and to act as a preservative.

The suspension was centrifuged and the rubbery residue resuspended in 5 volumes of buffer by a brief homogenization. After extraction in the cold with continuous agitation for 12 hr, the cycle of centrifugation, resuspension, and extraction was repeated at 24-hr intervals in 5 volumes of fresh buffer. This program was continued until the supernate no longer contained TCA-precipitable protein. At this stage, 5 volumes of distilled water were substituted for the buffer solution and extraction was continued as before, until no TCA-precipitable protein was present in the supernate.

The insoluble residue was suspended in 10 volumes of distilled water and autoclaved at 30 psi for 6 hr (19). This treatment extracts collagen as gelatin. The residue was washed once with boiling water (again resuspension was accomplished with the homogenizer), and the cycle of autoclaving and washing repeated with fresh distilled water, until the supernatant from the autoclaving was peptide-free as determined by the Biuret reaction (20). The residual material (autoclave-prepared elastin) was dehydrated as before, extracted with ether, and air-dried. Representative portions were oven dried for the determination of residual water. Other portions were taken for determination of ash (21) and nitrogen. Further duplicate portions of this elastin residue were treated with hot 0.1 N NaOH as described above, and the alkali-insoluble residue was determined gravimetrically.

AMINO ACID ANALYSIS: Samples of protein were hydrolyzed under nitrogen in 6 N HCl in sealed ampoules at 117°C for 72 hr. This period of hydrolysis is necessary for complete hydrolysis of insoluble elastin. Complete amino acid analysis was performed using the 22-hr Technicon column and gradient system as recommended by the manufacturers (Technicon Instruction Manual, Technicon Co., Chauncey, N.Y.). All analyses were performed in duplicate, and the results were reproducible to better than ±2%. Proline was determined separately by the method of Troll and Lindsley (22), and hydroxyproline was determined by the Stegmann procedure, as modified by Woessner (23).

DETERMINATION OF COLLAGEN: The supernatants from the autoclaving and washing procedures, containing the extracted gelatin, were pooled and a measured volume was dried on a steam bath. This was taken up in 6 N HCl and hydrolyzed, in vacuo, for 16 hr at 110°C in an hydrolysis block. The hydroxyproline content was determined as above and converted to the corresponding collagen value by assuming the hydroxyproline content of mammalian collagen to be 14.4%. (See Jackson and Cleary, 24).

OBSERVATIONS

Extraction Procedure

Several findings relating to the extraction procedure are noteworthy.

1. With the Tris-saline buffer the number of extractions required to produce a protein-free extract varied with the age of the ligament sample. For ligaments from animals in the last 2 fetal months, 12–13 extractions were necessary, but for ligaments on either side of this period fewer extractions were needed. In the 110-day fetus, the number of extractions had fallen to eight, and a similar number was required with ligament from a 14-day calf. In animals 3 months postnatal and older, the number of extractions required was five or six.

2. A similar pattern of responses was found in relation to the subsequent washing and extraction with distilled water. The initial water extract from the 9-month fetal ligament was heavily opalescent and contained TCA-precipitable protein. Further protein was extracted from this tissue in two subsequent extractions. As before, from younger and older ligament samples less protein was extracted over fewer extraction periods.

3. The nature of the protein in the material from these latter extracts was investigated briefly. With TCA, protein was precipitated from the pooled 11th, 12th, and 13th Tris-saline extracts of a 9-month fetal ligament. It sedimented as a white precipitate, which was extracted with ether to remove the TCA and then with acetone and finally air-dried to a fine light-brown powder. This material had a nitrogen content of 12.6%. Its amino acid composition was similar to that of the material extracted with strong salt solution from autoclaved adult elastin by Gotte et al. (25) and to that of the ground substance proteins isolated by Partridge et al. (26) and by Barnes (27) (See Table I). As the amino acid pattern showed a high content of

TABLE I

Amino Acid Composition of the Ground Substance Proteins from Various Tissues

Amino acids	Grams of Amino acids/100 g dry ash-free tissue			
	Ligament*	Ligament elastin Gotte et al. (25)	Aorta Barnes (27)	Nasal cartilage Partridge et al. (26)
Cysteic acid	1.13	1.37	1.0	
Hydroxyproline	0.75	2.75	<0.2	<0.2
Aspartic acid	6.1	6.44	9.3	6.54
Threonine	2.04	3.03	4.2	2.52
Serine	1.36	4.45	4.3	3.00
Glutamic acid	8.14	10.28	11.8	9.08
Proline	12.0	7.38	5.5	5.10
Glycine	3.9	10.43	3.9	5.00
Alanine	2.91	6.25	4.1	3.40
Valine	3.59	5.8	5.2	4.20
Methionine	0.84	1.24	2.2	1.20
Isoleucine	2.65	4.02	4.6	3.10
Leucine	5.15	7.02	7.9	7.20
Tyrosine	1.86	1.23	5.6	3.50
Phenylalanine	2.69	3.54	5.0	3.95
Lysine	3.37	4.19	5.5	3.63
Histidine	1.28	1.46	2.2	1.73
Arginine	4.18	4.01	6.2	5.30
Nitrogen	12.6	13.25	14.93	11.81

* No correction has been made for amino acid losses due to hydrolysis.

polar amino acids and a low content of glycine and valine, this protein can contain very little, if any, elastin. Therefore, this line of investigation was not pursued further.

Results of Chemical Analyses

Fig. 1 shows the pattern of changes in the concentrations of water and DNA in the ligament during its development. The degree of hydration remains high throughout the first 7 fetal months, at which time it begins to fall. It falls rapidly during the last 2 months of pregnancy and through the 1st postnatal month, but therefter the values level off slowly, so that the adult level has been attained by the age of 3 months. There is little further change, even in the oldest samples available (10 yr).

The DNA concentration of the fresh ligament can be seen to undergo a linearly progressive fall throughout pregnancy. After birth the DNA concentration quickly levels off, and this value is maintained into adult life. When allowance is made for the variation in water concentration, and the results are expressed in terms of the dry weight of the ligament, the pattern of changes in the concentration of DNA is little altered. The linear decline throughout pregnancy is still seen, but occurs at a greater rate. The adult level is reached at about the same time, during the 2nd month postpartum.

Fig. 2 shows the changes in the concentrations of collagen and alkali-insoluble elastin during development. The values, in each instance, are expressed in terms of the dry weight of the tissue. It can be seen that the elastin concentration remains quite low until about the end of the 7th fetal month. Subsequently, the concentration of elastin increases dramatically over the last 2 months of pregnancy and during the 1st postnatal 2 wk, by which time it has reached the adult levels.

Fewer estimations are available for collagen, but they are sufficient to establish that the concentration of collagen rises earlier and is greater than that of elastin until late in pregnancy. Then, over a short time interval, it falls to the adult levels, which are about half the late pregnancy values.

There are considerable differences in the values obtained for the insoluble elastin concentrations of the younger ligaments, by the use of the two

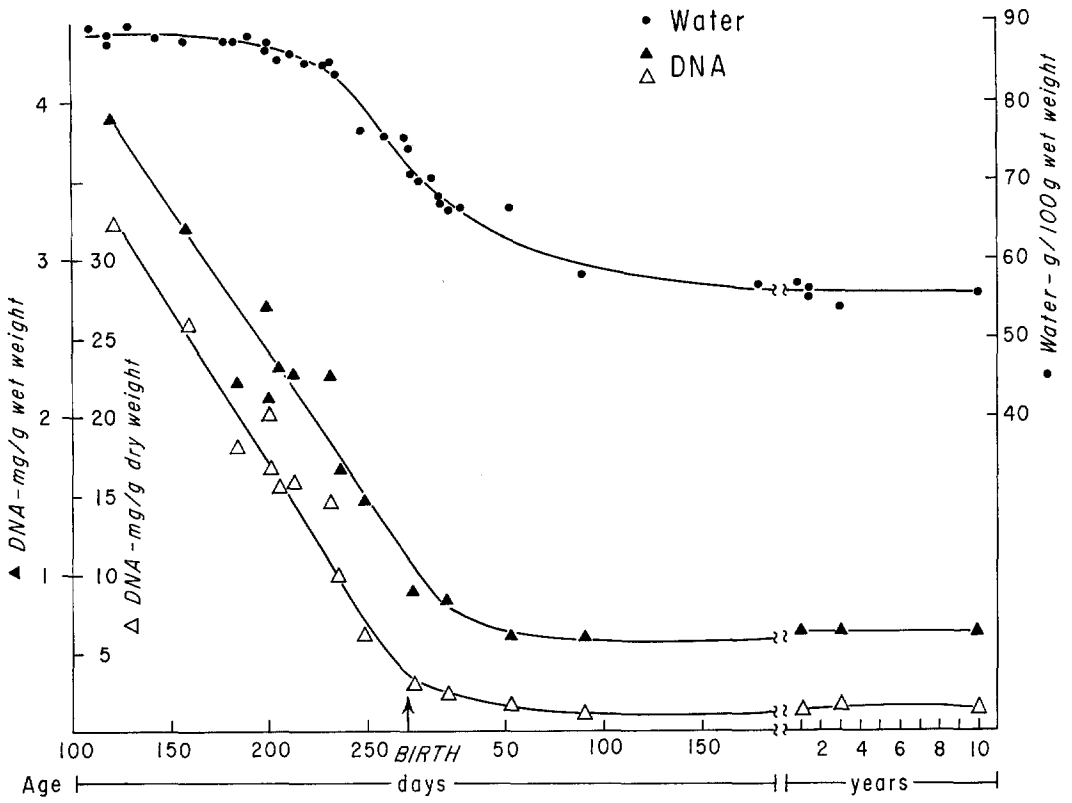

FIGURE 1 Changes in water and DNA concentration in bovine nuchal ligament during development.

different extraction methods, as can be seen also from Fig. 2. It is unfortunate that in the young fetus the two measurements were not made on the same ligament samples, but this was not done because all the tissue was used for the original extractions. Nevertheless, it is apparent that the yield of elastin by the autoclave method of extraction is much higher than that of elastin obtained by alkaline extraction. Towards the end of the period of intrauterine development, the difference in the yields from the two methods of preparation becomes less marked, so that in ligament samples from the term fetus there is some 15% less elastin residue from the alkaline method than from the autoclaving method. In the postnatal samples, this difference has fallen to 7% at 18 days of age. In the adult ligaments, the difference has become insignificant, the alkaline extraction method giving values about 1–2% lower than those from the autoclaving procedure.

Amino acid analyses of the autoclave-prepared elastin residues have shown that, while the amino acid composition of the adult samples is virtually identical with that reported by Partridge and Davis (28) for similar materials, the fetal residues have a significantly increased content of polar amino acids. This has been interpreted as indicating a noncollagenous protein contamination. In the younger fetal ligaments, the extent of this contamination is considerable. The alkaline extraction procedure yields a more elastin-like product, although even this method does not completely "purify" the elastin from these young tissues. The detailed findings of this work have been reported elsewhere (29).

Quantitative Changes in Ligament Composition

Fig. 3 shows the changes in the ligament weight during pregnancy and the 1st 2 postnatal months. The dry mass of the ligament increases only slowly until the 8th month of pregnancy, at which time it increases rapidly. The weight of the ligament continues to rise, but at a slowly decreasing rate throughout the remainder of the observation period (up to 53 days after birth). Unfortunately

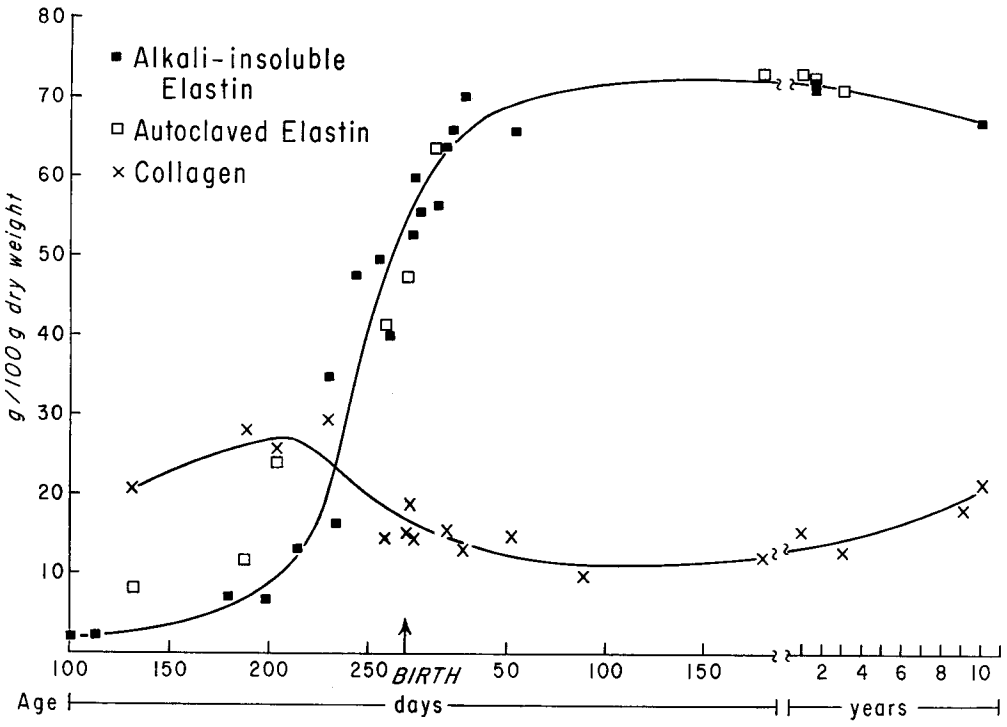

FIGURE 2 Changes in concentration of collagen and elastin in bovine nuchal ligament during development. The elastin values shown are those for the alkali-insoluble residue.

whole ligaments could not be obtained from animals older than 53 days.

The pattern of changes in the total DNA content of the whole ligaments is shown in Fig. 4. There is a logarithmic-type increase in the amount of DNA per ligament throughout pregnancy. In the immediate postnatal period, however, there is a sharp fall in the total DNA content and this continues for the remaining 2 months of the study. The validity of this latter observation, which is based on very few actual determinations, can be reinforced by calculating the DNA content of the other ligaments in this period (i.e., those for which the total weights are known, but for which reliable DNA analyses are not available). This has been done by reading values for their DNA content from the graphs in Fig. 1. On the basis of the dry weight, as the observed values fit a smooth curve with very little scatter the likely maximal error induced would be small and so would not effect the observation significantly. The values determined in this way for the total DNA content of the additional ligaments are shown separately in Fig. 4 (indicated by △). It will be seen that these additional points conform closely to the observed pattern.

Fig. 5 shows the values for the total collagen and elastin contents of the whole ligaments during the period for which quantitative measurements were possible. The collagen content is still quite low until the end of the 6th fetal month, at which time it begins to increase more rapidly. This increased rate of collagen deposition continues steadily throughout the next 4 months.

In view of the previous observations on the amino acid analysis of the elastin residues obtained by the different extraction methods, the alkali-insoluble residue has been used for determining the total elastin content. It will be seen that the elastin mass remains very low until the end of the 7th month of pregnancy when it begins to increase more rapidly. By the end of the 8th month of pregnancy, a phase of extremely rapid elastin formation commences which continues, without noticeable slowing, throughout the period up to the age of 2 months. The approximate rate of insoluble-elastin formation during this period is between 0.25 and 0.3 g per day. In the absence of

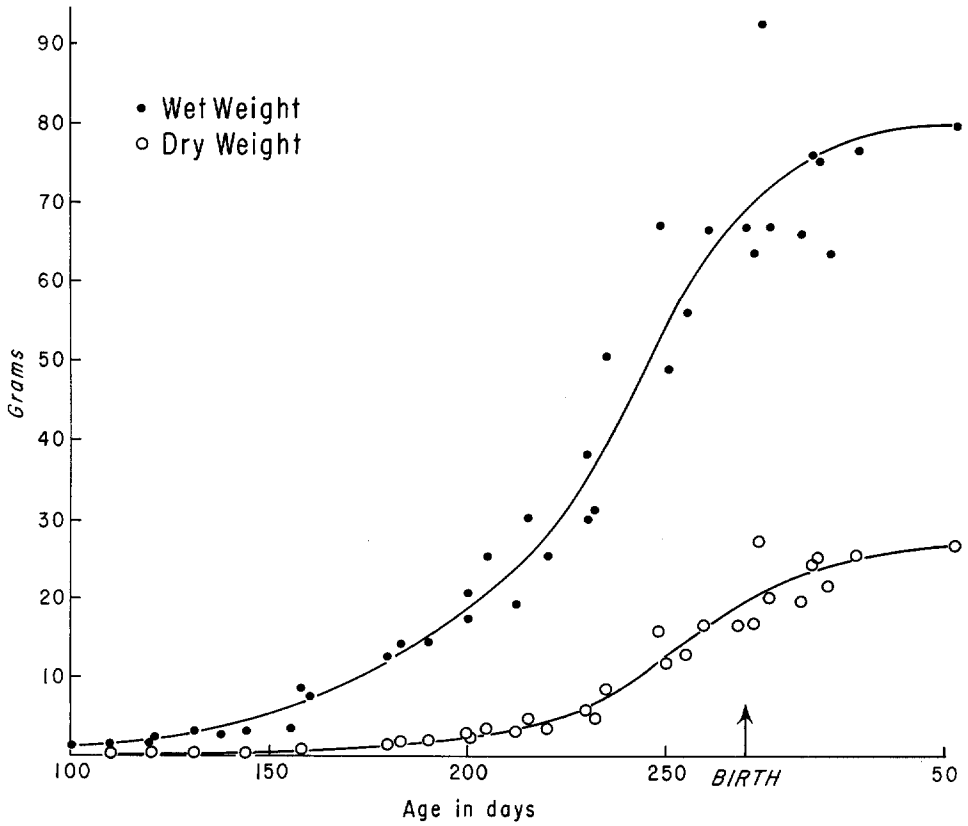

FIGURE 3 Values for fresh and dry weights of whole bovine nuchal ligaments, during gestation and the early postnatal weeks.

data on the subsequent changes in the ligament weight, it is not possible to determine accurately when elastin deposition decreases.

DISCUSSION

The findings indicate that the bovine nuchal ligament conforms in many ways with the development pattern usually observed in other organs. Collagen is present in appreciable concentrations in quite young fetal ligaments and its appearance precedes the development of elastic fibers, as has been reported in other tissues (30). In common with other fetal tissues, the ligament is initially highly hydrated and composed predominantly of cells. Subsequently, the degree of hydration falls; this can be related to the increasing content of fibrous proteins and the reduction in cell concentration.

Extraction Procedure

In an earlier study, which included the human fetal aorta (31), it had been suspected that the elastin values obtained with the alkaline extraction procedure were unexpectedly low when compared with the histological appearances. This was considered to indicate the probability that the elastin in the fetus was more susceptible to solubilization than was older elastin. A similar discrepancy has since been reported for the human pulmonary artery (32). It was the initial intention, therefore, to attempt to prepare and quantitate the elastin of the ligaments by the less severe method of repeated extraction and autoclaving. This method had been used by Partridge and Davis (28) who found it to yield, from adult bovine nuchal ligament, an elastin residue which had an amino acid composition comparable to that of elastin obtained by the alkaline extraction procedure.

In employing this procedure, several important modifications were introduced:—

a. The buffer used for the extractions was that which had been shown previously to give maximal swelling and extraction of protein

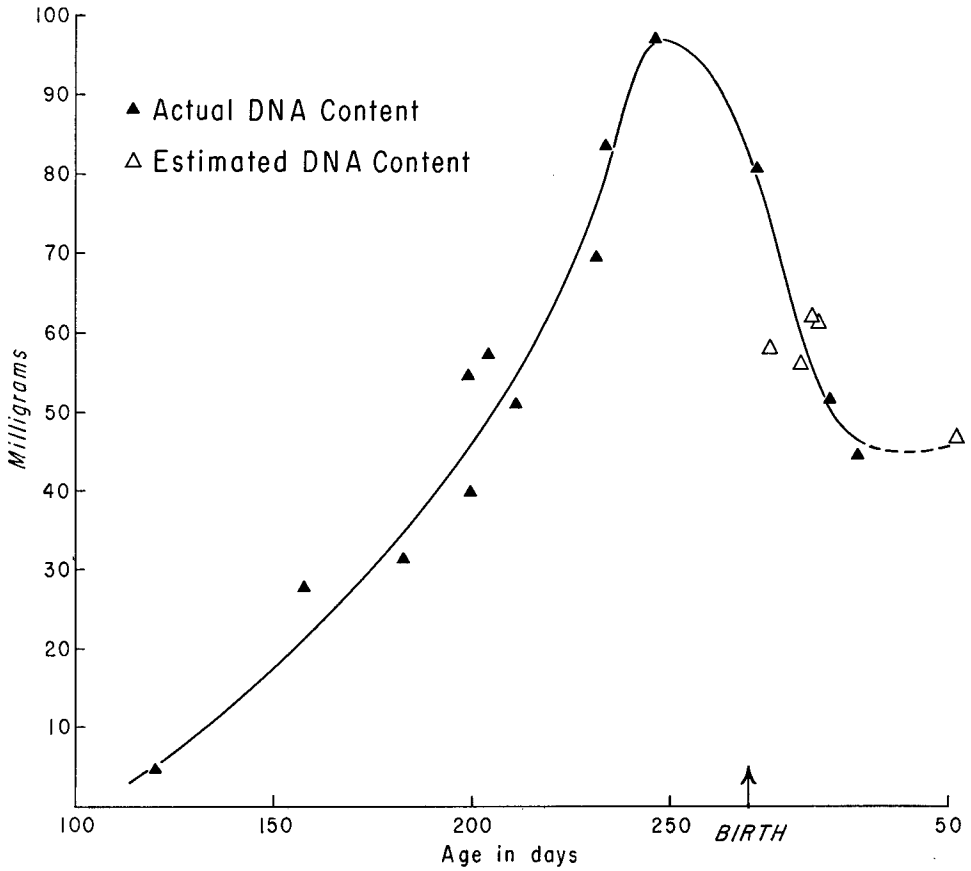

FIGURE 4 Changes in total DNA content (cellularity) of the developing bovine nuchal ligament. In the early postnatal period, several values have been calculated using values for DNA concentration read from Fig. 1. These are indicated separately (△).

from ligaments of various ages (11);

b. Adequate fragmentation and mixing was assured at each stage in the preparation by a brief period of homogenization; and

c. The autoclaving routine was altered, as it was felt likely that the drying and grinding process used by Partridge et al. (1) may induce further denaturation of the contaminating protein and so render extraction of it more difficult. For this reason, autoclaving was performed at 30 psi over 6-hr periods, as recommended originally by Lowry et al. (19).

That the residual material from adult ligament had an amino acid composition comparable to that of alkali-insoluble elastin attests to the reliability of the modified procedure with tissues of this age. However, despite the additional precautions, the product obtained from fetal samples showed significant contamination with a noncollagenous protein having a high content of polar amino acids and also with nonnitrogenous materials. Rather surprisingly, these contaminating materials were not completely removed by a subsequent alkaline extraction (29).

Alkaline extraction, without prior autoclaving, yielded a residue more closely resembling elastin than did either of the other procedures, especially in the case of the fetal ligaments, and for this reason it was finally chosen as the procedure for the quantitative study. The yield of elastin from growing ligaments was least by this method, although with mature ligaments all three extraction procedures gave similar yields.

Cellular Activity

As the DNA content of diploid cells is constant (33), the ligament DNA content may be considered an index of cellularity. If the vascular-

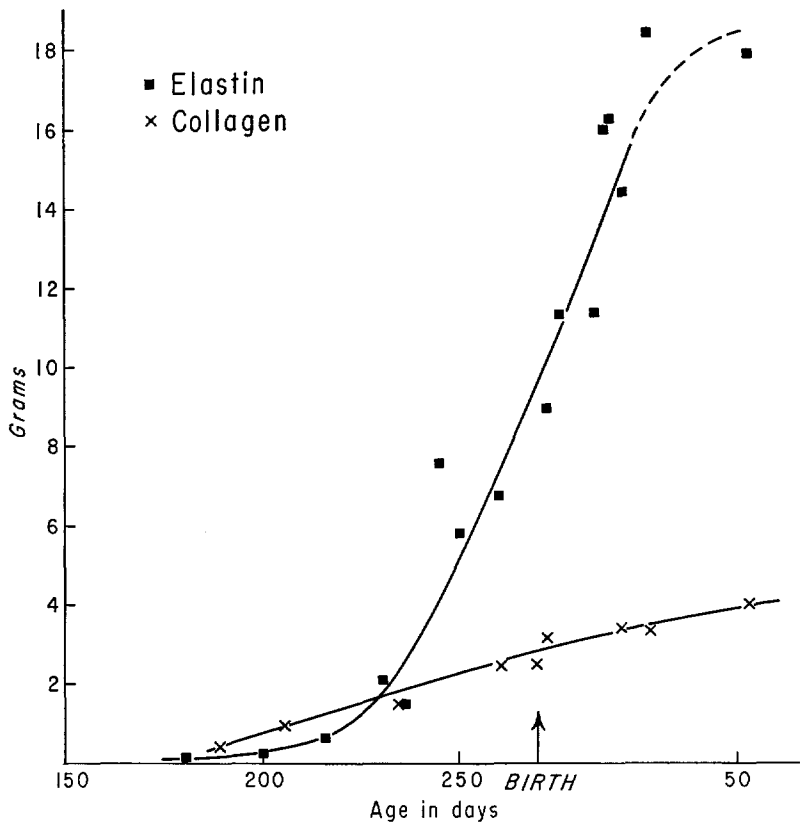

FIGURE 5 Changes in the total amounts of collagen and alkali-insoluble elastin in the bovine nuchal ligament during development.

related cells are excluded, DNA content will then be a measure of the fibroblast population. Histologically, it has been shown (12) that the vascular-related cells account for only a small proportion of the total ligament cellularity, even in the 180-day fetus when ligament vascularity is maximal. As vascularity falls rapidly in the older ligaments (so that at birth vessels are seen only infrequently in ligament sections), it is apparent that the presence of vascular cells will not significantly alter the interpretation of the changes in ligament fibroblast population based directly on the DNA estimations.

The changes in ligament DNA concentration (Fig. 1), which indicate a progressive fall in cell population density throughout fetal development, are similar to those in other organs. However, when the changes in total cell number per ligament are compared with the changes in the total insoluble elastin content, a surprising temporal dissociation is seen, in that elastin deposition is just beginning to increase very rapidly at the time when the total cell number per ligament is already maximal. Subsequently, this rapid rate of elastin deposition is maintained although the total cell number is falling. This may mean either that the individual cells increase their rate of elastin synthesis to take over the extra load as other cells regress, or alternatively that the number of cells involved in elastin synthesis remains constant while nonsecretory cells disappear.

Furthermore, it has been seen (Fig. 5) that during fetal development the rates of production of collagen and elastin alter not only absolutely but also in relation to one another. How these changes are brought about is not clear, but the matter is further complicated by the electron microscopic finding (7) that individual fibroblasts in these ligaments are apparently producing both collagen and elastin simultaneously, rather than that certain cells are responsible for the production of one or the other material.

One surprising finding has been the absence of any demonstrable alteration in either the rate of production of collagen and elastin or the total ligament mass, concomitant with the major alterations in stresses and functional activity that must be attendant upon birth and the need for the ligament to provide support for the head. Although the decline in ligament total cellularity takes place at or soon after birth (Fig. 4), it has not been possible to establish whether this is a fortuitous or causal relationship.

Elastin Synthesis and Alkali-Insoluble Elastin

It has been necessary to distinguish between alkali-insoluble elastin content and the rate of elastin synthesis. Until the nature of the cross-links responsible for conferring alkali-insolubility on elastin are understood, or until tropoelastin has been isolated, one can only speculate on the duration of the time-lag between the secretion of soluble elastin by the cells and its deposition within the alkali-insoluble core of the elastic fibers in the growing ligament. Experiments[2] with aortas of young growing chicks, in vivo, have shown that there is significant incorporation of lysine-^{14}C into the alkali-insoluble elastin, within 4 hr of intravenous injection of isotope. This indicates that only a short time interval is involved between synthesis and deposition in this tissue. If this is true of the nuchal ligament, the relationship of the cell changes to elastin synthesis will be the same as that of the cell changes to the deposition of alkali-insoluble elastin.

The decision as to which age period is likely to be the most suitable for the isolation of maximal amounts of tropoelastin can be made with reasonable chance of accuracy, on the basis of these findings. The total mass of elastin per ligament is increasing most rapidly between 225 days of gestation and the end of the 1st month postpartum. At about this same time, the concentration of elastin in the ligaments begins to rise, and it, too, levels off at about the end of the 1st postnatal month. As the cell concentration and the total cell content are still appreciable in the prenatal period, it may prove preferable to avoid this stage. At the end of the 1st postnatal month the elastin production may be beginning to fall off, and thus it would be advisable not to look too late in this phase, especially as the interval between synthesis and alkali-insolubility is unknown. It seems likely, however, that this interval will be measured in hours or, at most, days, rather than in weeks. As a compromise with respect to all these factors, the period of 2–3 wk immediately following birth would seem to offer the greatest promise in the search for a soluble elastin precursor from bovine ligament.

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