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QUANTITATIVE INTERRELATIONSHIPS OF THE CHIEF COMPONENTS OF SOME CONNECTIVE TISSUES DURING FOETAL AND POST-NATAL DEVELOPMENT IN CATTLE*

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INTRODUCTION

In the course of its foetal and post-natal development the intercellular substance of connective tissue undergoes certain changes which are sometimes called maturation. Histological studies have been concerned chiefly with the morphology of the fibrous material, which as a rule has a network-like appearance in the early stages, the fine fibres having a strong affinity for complex silver salts (reticulin). This structure may change gradually, via several intermediate forms, into a coarser, stiffer, bundled structure, which is only very slightly argentophil (collagen). This maturation is not a uniform process that takes place in the same way in all connective tissues. Sometimes the reticular type is maintained (reticular connective tissue) and sometimes the

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collagenic type predominates even in young individuals (*e.g.* in tendons). Sometimes again the histological pattern is so far removed from the accepted criteria that it cannot well be fitted into this nomenclature (cornea).

HERINGA AND HOOFT[1] noted that environmental factors influenced the aspect of the fibrous material. They found that the same fibre was argentophil in the neighbourhood of the cells and not so elsewhere.

Between the cells there lie not only fibres, but also the amorphous, homogeneous ground substance. The two components are physico-chemically related to each other[2,3]. Starting from the hypothesis that the morphological picture is a reflection of the chemical and physico-chemical relationship between the components of the tissue in question, we found it necessary to make a quantitative study of the proportions in which the chief components of the intercellular substance occur, as a preliminary to further investigation. Such analyses had already been done for a number of tissues of adult cattle, but a comparison of the different tissues is vitiated by certain factors not yet accessible to investigation which may be referred to as the 'specific nature' of each tissue. We therefore undertook a chemical investigation of the embryonal and post-natal *development* of certain tissues, each considered separately. This investigation originally consisted of determinations of collagen and mucopolysaccharides, which are generally regarded as the chief components of connective tissue. At a later stage of the investigation we also determined the amounts of non-collagen proteins, which form a quantitatively important fraction of certain connective tissues, as shown by HOOGHWINKEL[1].

MATERIAL AND METHODS

The choice of connective tissues for investigation was guided by the following considerations:

(a) The tissues must be such that they can be dissected out in a reproducible manner.

(b)The tissues must be as 'homogeneous' as possible in structure, *i.e.* the different parts of a sample must differ as little as possible in structure and composition.

(c)The material must be available in sufficient quantity without the use of other than mechanical methods of isolation.

The tissues chosen were cornea, sclera, Achilles tendon, and skin, of bovine embryos, newborn calves and adult cattle. Costal cartilage and articular cartilage were at first also included in the investigation, but they yielded such variable results within one and the same age-group that no comparison of different age groups was possible. This was probably due to the difficulty of isolating samples in a reproducible manner.

The bovine embryos* were stored at 0–5° C. The tissues were dissected out about 24 h after death. The length of the embryos was obtained by bringing the neck into a position in a line with the spine and measuring the distance from the lower end of the spine to a line tangential to the skull and perpendicular to the spine.

Cornea and sclera were dissected with an ample margin from the limbus, the eyes having first been carefully freed from fat and connective tissue. After isolation, the tissues were dried with a piece of filter paper.

Achilles tendon was wiped clean superficially and further used in its entirety. Flaps of skin were cut from the backs of the embryos and freed from the easily removable epithelium and fat by scraping with a blunt knife. We were unable to obtain pieces of skin from the backs of the newborn calves and adult cattle, on account of the loss of economic value of the hides that this would have involved. Instead we took pieces from the outside of the upper part of the fore-limbs.

Immediately after isolation the fragments of tissue were dehydrated in acetone at room temperature. After several days at least, during which time the acetone was renewed several times, the fragments were placed over $CaCl_2$ in a desiccator, in which they were further kept. Before the analysis they were dried *in vacuo* for 3 h at 105° C.

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For hexosamine determinations the following modification of the method of ELSON AND MORGAN[5] was used: A suitable amount of dried tissue was hydrolysed with 5 ml of 2*N* HCl for 16 h at 100° C. Solid $CaCO_3$ was added to the hydrolysate until no more gas was evolved and then Ca^{++} was precipitated with 3 ml of a saturated solution of potassium oxalate. After shaking, the mixture was centrifuged: 2 ml of a mixture of 25 ml 0.5 *N* Na_2CO_3 and 0.75 ml freshly-distilled acetylacetone were added to 2 ml of the supernatant, and thoroughly mixed, the tube was closed with a rubber stopper and heated 20 min in a boiling waterbath. After the contents of the tube had cooled to room temperature, 4 ml of 96% alcohol were added and then 2 ml of a solution of 1.6 g *p*-dimethylaminobenzaldehyde in 30 ml of a 1:1 mixture of 96% alcohol and 36% HCl, cautiously added. The whole was allowed to stand for 45 min and the extinction was read at 530 mμ in a Coleman Junior spectrophotometer. The amount of hexosamine-HCl present was read from a standard curve obtained by the same method. A determination on a standard solution of hexosamine was run as a check with each analysis.

Hydroxyproline was determined by the method of NEUMAN AND LOGAN[6] and tyrosine by that of UDENFRIEND AND COOPER[7].

Calculation of percentages of collagen and mucopolysaccharides

The percentage of collagen was calculated from the value found for hydroxyproline. Collagen in the chemical sense (chemocollagen) is a protein that is characterised by a high hydroxyproline content (the most probable value for mammalian collagen is 13.6%[8,9,10]. Reticulin from fat tissue and lymph nodes, which is regarded as a collagen type of protein[11,12], was found by BOWES AND KENTEN[13], who used a semiquantitative paper-chromatographic method, to have a slightly lower hydroxyproline content than ox-hide collagen. We calculate the chemocollagen content by multiplying the hydroxyproline content by a factor of 100/13.6; this means that we assume that reticulin contains a small amount of another substance[14,15] in addition to chemocollagen. Hydroxyproline does not occur in measurable amounts in any other protein[16], except perhaps elastin. According to NEUMAN[17], elastin contains 2% of hydroxyproline, but DEMPSEY AND LANSING[18] are of the opinion that this may be due to collagen included in the elastin. In view of the relatively small amount of this protein in the tissues examined by us, the interference caused by it in the collagen determination can be regarded as negligible.

The mucopolysaccharide content was calculated from the value found for hexosamine-HCl. The most important mucopolysaccharide components — in the quantitative sense — of the tissue examined are chondroitinsulphuric acid and keratosulphate. The contain respectively 47.1 and 48.05% of hexosamine calculated as hydrochloride. Multiplication by a factor of 2.1 gives a reasonably good approximation to the mucopolysaccharide content[4].

Part of the material was also analysed for tyrosine. HOOGHWINKEL[4] had found that non-collagen proteins obtained by extraction from tissues had a much higher tyrosine content than chemocollagen (5.5 against 0.6%). From these figures an estimate of the proportion of non-collagen protein (NCP) was made as follows:

$$NCP(\%) = \frac{T - HP \times \frac{0.6}{13.6}}{5.5} \times 100$$

where T = tyrosine found and HP = hydroxyproline found.

RESULTS

For the sake of clarity in the presentation of results, the foetal material has been divided into a number of groups. These groups have been so chosen that the number of observations per group is sufficient for statistical analysis while at the same time the characteristics of the curves, as seen when the total of results is plotted, are fully preserved.

When part of the data on hexosamine content of cornea, sclera and tendon had already been obtained, we decided to include skin in the investigation and to determine hydroxyproline contents. The number of such observations is thus smaller than the number of hexosamine determinations, but is still large enough to warrant inclusion in the discussion. Tables I and II show the mean values for hexosamine-HCl, hydroxyproline and tyrosine (in percentages of dry weight) calculated for the various

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TABLE I

HEXOSAMINE-HCl AND HYDROXYPROLINE DETERMINATIONS (% OF DRY WEIGHT) IN CORNEA, SCLERA, ACHILLES TENDON AND SKIN OF FOETUSSES, NEWBORN AND ADULT CATTLE

n = number of observations, σ_m = standard error of the mean

Groups	Cornea						Sclera						Achilles tendon						Skin (back)					
	hexosamine-HCl			hydroxyproline			hexosamine-HCl			hydroxyproline			hexosamine-HCl			hydroxyproline			hexosamine-HCl			hydroxyproline		
	n	mean	σ_m	n	mean	σ_m	n	mean	σ_m	n	mean	σ_m	n	mean	σ_m	n	mean	σ_m	n	mean	σ_m	n	mean	σ_m
Foetusses																								
10½–15 cm	6	2.49	0.12	5	5.1	0.7	6	1.66	0.09	4	4.7	0.4	6	1.90	0.07	4	4.0	0.5	5	3.01	0.15	5	1.5	0.1
15½–20 cm	17	1.79	0.04	15	5.7	0.2	18	1.20	0.03	17	6.4	0.2	16	1.32	0.02	17	4.6	0.2	17	2.22	0.06	17	1.7	0.1
20½–25 cm	13	1.79	0.05	10	6.8	0.2	13	1.06	0.02	13	7.1	0.1	13	1.20	0.02	12	5.8	0.2	13	1.62	0.03	13	2.1	0.1
25½–30 cm	16	1.83	0.06				15	0.96	0.02				16	1.13	0.03									
30½–35 cm	11	1.93	0.05	8	7.8	0.2	11	0.90	0.03	9	8.3	0.2	11	1.04	0.04	9	6.9	0.2	9	1.23	0.06	9	2.6	0.2
35½–40 cm	13	1.95	0.04				13	0.86	0.03				13	1.03	0.03									
40½–50 cm	14	2.13	0.03	6	8.4	0.2	14	0.78	0.02	6	8.9	0.1	14	0.94	0.03	6	8.5	0.4	6	1.14	0.13	6	5.0	0.2
50½–70 cm	19	2.22	0.04				19	0.67	0.01				19	0.78	0.03									
Newborn calves	17	2.28	0.03	17	7.7	0.1	17	0.54	0.01	17	10.0	0.2	17	0.54	0.01	17	10.6	0.2	15	0.55	0.007	15	7.4*	0.2
Adult cattle	15	2.32	0.03	15	7.2	0.1	15	0.50	0.01	15	10.3	0.2	15	0.26	0.01	15	10.7	0.2	15	0.53*	0.008	15	8.9*	0.3

* Skin of the legs.

TABLE II

TYROSINE CONTENTS OF CORNEA, SCLERA, ACHILLES TENDON AND SKIN OF THE LEGS OF NEWBORN AND ADULT CATTLE

n = number of observations, δm = standard error of the mean

	Newborn calves			*Adult cattle*		
	n	*mean*	σ_m	n	*mean*	σ_m
cornea	17	1.64	0.03	15	1.93	0.03
sclera	17	1.10	0.02	15	1.13	0.02
tendon	17	1.03	0.01	15	0.86	0.01
skin of the legs	15	1.76	0.05	15	1.27	0.04

groups, in addition to the number of observations per group and the standard error of the mean.

The general tendency is that hexosamine decreases and hydroxyproline increases with age. Sclera, tendon and skin all show the same pattern, as represented in Fig. 1. The length of the embryos is shown on the horizontal axis; the newborn calves were not measured, but their position on the axis corresponds to the lengths reported in the literature[19].

During early embryonal development the drop of hexosamine content in skin is somewhat steeper than that in tendon and sclera. The rise of hydroxyproline con-

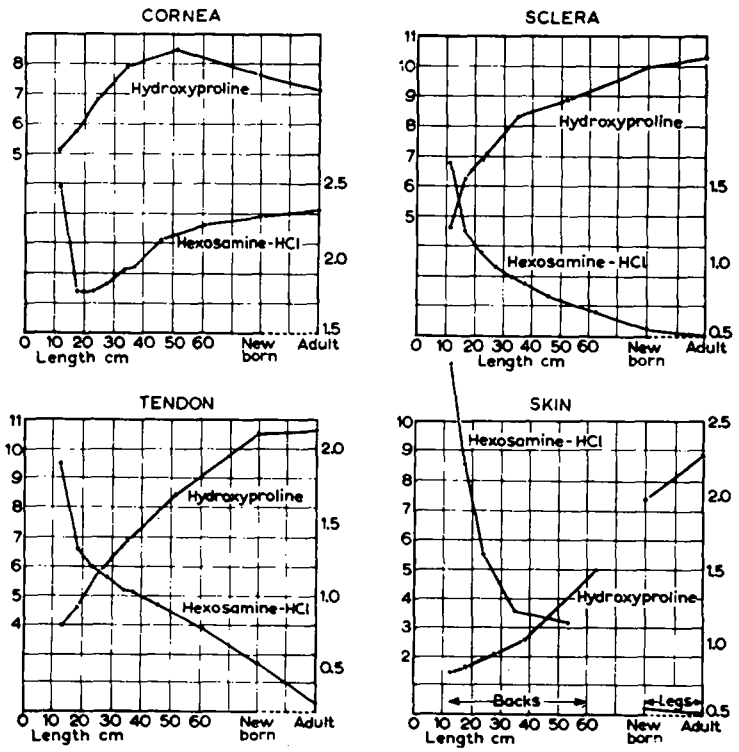

Fig. 1. Hexosamine-HCl (right axis) and hydroxyproline (left axis) concentrations in % of dry weight in some connective tissues of cattle in the course of the development.

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tent in sclera occurs at an earlier stage than in skin, while the rise in tendon proceeds at a practically uniform rate throughout foetal development. After birth the hexosamine content of the skin of the limbs remains about the same, but in sclera we observed a significant decrease ($P < 0.05$) and in tendon a highly significant decrease ($P \ll 0.01$). The hydroxyproline content of tendon and sclera remains practically unaltered after birth, but in the skin of the limbs it rises significantly ($P < 0.05$). HERINGA[20] also observed an appreciable further increase of the collagen character of the fibrous material in the skin of cattle after birth. As shown in Table II, the rise of the hydroxyproline content of skin after birth is accompanied by a significant fall of the tyrosine content.

Cornea differs markedly from the other three tissues in its development. In the very early embryonic stage it does indeed also show the decrease of hexosamine and increase of hydroxyproline, but then it seems as though a new phase appears in the mucopolysaccharide metabolism. The hexosamine content begins to rise and in the group of 30.5–35 cm length it is already significantly higher than in the groups between 15.5 and 25 cm.

Cornea also stands apart as regards the development of its collagen component. The hydroxyproline content at first rises, as in other tissues, but towards the end of gestation it begins to fall; the decrease is already significant in the newborn animal

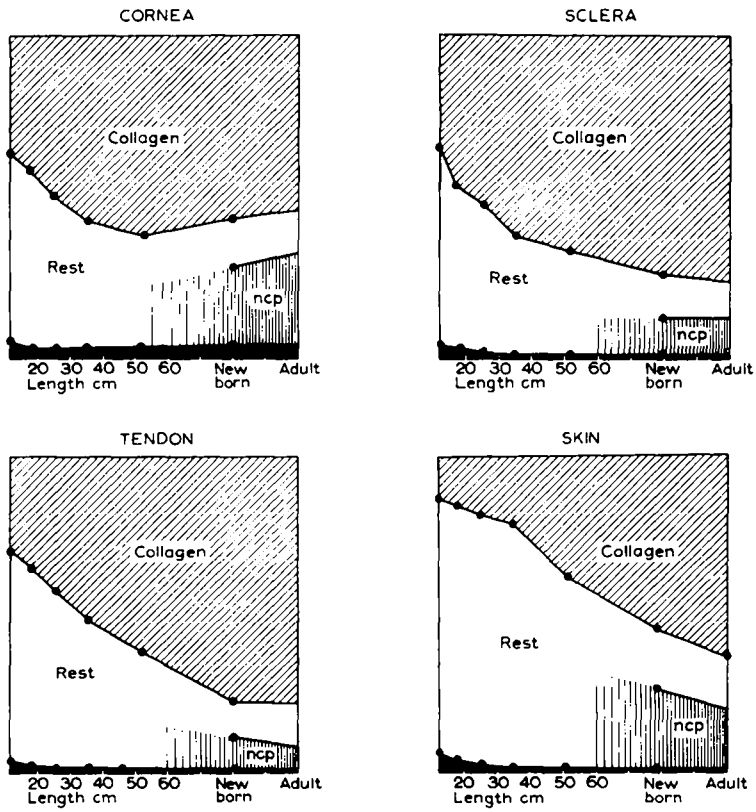

Fig. 2. Proportions of the chief components of some connective tissues of cattle in the course of the development. N.C.P. — non-collagen-protein.

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and continues after birth, so that the difference in hydroxyproline content between corneae of newborn and adult animals is significant. This is accompanied by a significant rise of the tyrosine content (Table II).

To show the quantitative interrelationships between collagen, mucopolysaccharides and non-collagen proteins in the tissues studied, the values calculated for these components in percentage of dry weight are given in Fig. 2. Three features call for comment:

(1) The quantitative significance of the mucopolysaccharides is only small. This is remarkable in view of their qualitative importance with regard to water-binding and the transparency of corneal tissue[21].

(2) Bovine skin is still a relatively collagen-poor tissue in the early foetal stage and always remains lower in collagen content than tendon and sclera.

(3) Although after birth the collagen content of the cornea decreases significantly and that of the skin increases significantly the proportion of non-collagen protein changes in the opposite direction, so that there remains a constant fraction of the total for the non-mucopolysaccharide-non-protein components (salts, nucleic acids, lipids, etc.).

SUMMARY

Determinations of hydroxyproline and hexosamine in cornea, sclera, tendon and skin of bovine embryos, newborn calves and adult cattle have been carried out. The results give a picture of the development of these connective tissues, particularly as regards their collagen and mucopolysaccharide contents. As a general rule the hexosamine content decreases in the course of development, while the hydroxyproline content increases. Cornea develops in a more complicated way: the hexosamine content decreases at first, but at a quite early foetal stage it starts to increase again, while the hydroxyproline content reaches a maximum towards the end of foetal life and the decreases again.

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