A histochemical study of sulphated glycosaminoglycans associated with the somites of the chick embryo

By M. J. O'HARE

From the Chester Beatty Research Institute, London

SUMMARY

A histochemical analysis has been made of sulphated glycosaminoglycans (mucopolysaccharides) associated with chick embryo somites before and after the onset of overt cartilage differentiation. The sulphated glycosaminoglycans were distinguished and resolved into different types by the use of alcian blue at low pH and alcian blue 'critical electrolyte concentration' staining combined with hyaluronidase digestion.

The newly formed somites are bounded on their dorsal, ventral, and medial surfaces by basement membrane material as they are delimited from the unsegmented paraxial mesoderm. Such epithelial basement membrane material, which was first detected in association with the epiblast/mesoderm boundary in the stage-4 embryo, was found to contain a major chondroitin sulphate A/C fraction and a minor chondroitin sulphate B fraction. The notochord sheath contained similar sulphated glycosaminoglycans.

Sulphated glycosaminoglycans were first detected between cells of the somite 'core' at stage 14 and were subsequently seen to accumulate around the cells of the developing sclerotome and later around cells of the dermatome; the myotome was devoid of such material at these stages (stage 14-20). These pre-cartilaginous sulphated glycosaminoglycans were also of the chondroitin sulphate A/C plus chondroitin sulphate B types.

In contrast, the matrix material of newly forming vertebral cartilage, which was first seen in the anterior region of stage 21 embryos, was distinguished by its lack of a hyaluronidase-resistant sulphated glycosaminoglycan component, and therefore presumably contained only chondroitin sulphates A/C. Much later in development (after stage 33) small amounts of sulphated glycosaminoglycan with the staining properties of keratan sulphate were found in the perichordal and subperichondrial regions of the vertebral cartilage.

INTRODUCTION

Somites of 2-5- to 3-5-day chick embryos have been shown to contain sulphate-activating enzymes (Lash, Glick & Madden, 1964) and to synthesize mucoprotein (Marzullo & Lash, 1967) prior to the onset of overt cartilage differentiation. Lash (1968) has suggested that the low levels of mucoprotein synthesis observed in these somites represents a 'genotypic' expression of the chondrogenic character of predetermined somite cells, which later becomes converted into an overt 'phenotypic' expression as cartilage matrix. According to this concept,
the activity of cartilage ‘inducing’ agents results in the stabilization of this pre-existing metabolic pattern.

The precise chemical nature of the glycosaminoglycan (mucopolysaccharide) component of this precartilaginous mucoprotein is, however, of importance. Synthesis of sulphated glycosaminoglycan is not confined to cartilage, but occurs in other mesodermal connective tissues (Dorfman, 1963; Brimacombe & Webber, 1964). In the cartilage matrix of higher vertebrates, the sulphated glycosaminoglycan (GAG) component consists of a major chondroitin sulphate A (4-sulphate) and C (6-sulphate) fraction (Barrett, 1968) and an age-dependent keratan sulphate fraction (Pedrini & Pedrini-Mille, 1968). In many other mesodermal connective tissues, chondroitin sulphate B (dermatan sulphate, CS-B) is present in addition to chondroitin sulphate A/C (CS-A/C) (Meyer, Davidson, Linker & Hoffman, 1956). Marzullo & Lash (1967) recovered sulphated GAGs from 2-5- to 3-5-day chick embryo somites, and identified a major CS-C fraction, but the presence or absence of a minor CS-B fraction in this material was not recorded.

CS-B differs from CS-A/C in that most of the glucuronic acid residues of the latter are replaced by iduronic acid (Cifonelli, Ludowig & Dorfman, 1958; Fransson & Rodén, 1967). As the hexosamine moiety of CS-B and of CS-A/C is sulphated acetylgalactosamine (Brimacombe & Webber, 1964), the enzymes involved in the conversion of glucosamine to acetylgalactosamine, and in the sulphation of acetylgalactosamine, are common to both CS-B and CS-A/C synthesis. As these are the enzymes which Lash (1968) has shown by the metabolism of \([^{14}C]\)glucosamine to be present in 2-5- to 3-5-day chick embryo somites, they do not specify the nature of the GAG component of the somite mucoprotein.

It is possible, however, to distinguish between the various sulphated GAGs by the use of histochemical methods, and therefore to determine whether the GAG component of sulphated mucoprotein synthesized by young somites differs qualitatively from that of cartilage matrix. This paper reports a histochemical analysis of sulphated GAGs associated with the somites of the chick embryo before and after overt cartilage differentiation.

**METHODS**

Embryos were staged according to Hamburger & Hamilton (1951) and fixed in Newcomer’s fluid (1953). Embryos from stage 2 to stage 30 were examined. After washing in absolute ethanol, the embryos were cleared in toluene and embedded in Paramat (G. T. Gurr Ltd.) and serial sections prepared at 8 μm and 30 μm. Sections of cervical vertebrae from stages 30–41 (7–16 days) were also prepared for evaluation of staining procedures.

Sections were stained for sulphated glycosaminoglycans as follows.
A. General sulphated glycosaminoglycan staining

The ionized sulphate groups of the GAGs were made visible by staining with alcian blue 8GX 300 (G. T. Gurr Ltd., and gift of I.C.I.) at low pH.

Dewaxed and rehydrated sections were rinsed in pH 0.5 hydrochloric acid solution and then stained for 30 min in 0.5% alcian blue 8GX in pH 0.5 hydrochloric acid. After staining, the sections were blotted free of excess stain (Lev & Spicer, 1964) and rinsed in pH 0.5 hydrochloric acid to remove unbound stain before dehydration, clearing, and mounting in DPX. Under these conditions, only sulphate groups will be ionized and thus capable of binding the cationic dye (Johnson, Johnson & Helwig, 1962; Lev & Spicer, 1964; Quintarelli, Scott & Dellovo, 1964a).

Sections were examined microscopically with a red filter (Wratten 25) in order to accentuate alcian blue staining.

As a check on the specificity of the alcian blue staining, some sections were stained with azure A in pH 1.5 glycine buffer (Szirmai, 1963), sulphate groups being revealed by dye metachromasia.

B. Differential sulphated glycosaminoglycan staining

The sulphated GAGs demonstrated by the alcian blue-pH 0.5 staining procedure were resolved into different classes by the ‘critical electrolyte concentration’ (CEC) method of Scott & Dorling (1965). Sections were stained in solutions of 0.1% alcian blue 8GX in 0.025 M acetate buffer (pH 5.8) plus 0.4, 0.6 or 0.8 M MgCl₂, for 1 h. Sections were rinsed in MgCl₂ solutions of the appropriate molarity before and after staining and examined microscopically in aqueous medium and after dehydration, clearing and mounting in DPX.

Owing to their different critical electrolyte concentrations (at which the anionic polymers change from binding dye to binding Mg²⁺), chondroitin sulphates A/C, chondroitin sulphate B, heparin and keratan sulphate will stain in 0.4 M MgCl₂ whereas only heparin and keratan sulphate will stain in 0.6 M MgCl₂ and only keratan sulphate will stain in 0.8 M MgCl₂ (Scott & Dorling, 1965).

C. Hyaluronidase digestion

To distinguish between chondroitin sulphate A/C and chondroitin sulphate B, sections were incubated with ovine testicular hyaluronidase (350–500 i.u./mg, B.D.H.) at concentrations of 0.2 mg/ml and 1 mg/ml for 4 h and 24 h at 37 °C in pH 6.0 citrate buffer before staining. Control sections were incubated in buffer alone.

Testicular hyaluronidase attacks the endo-β-N-acetylgalactosamine-glucuronic acid links in CS-A/C (Ludowieg, Vennesland & Dorfman, 1961). It does not attack acetylgalactosamine-iduronic acid links and therefore degrades CS-B very little (Fransson & Rodén, 1967) and may be used histochemically to
distinguish the labile CS-A/C from the resistant CS-B, heparin and keratan sulphate (Leppi & Stoward, 1965).

D. Chemical blocking

To check the specificity of the alcian blue staining for sulphated GAG, sections were treated with 0.1 N HCl in absolute methanol at 60 °C for 4 h. This treatment abolishes sulphate-specific staining (Quintarelli, Scott & Dellovo, 1964b).

RESULTS

Sulphated GAGs have been found in association with the ectodermal, endodermal, neural and notochordal surfaces of the somite as a component of basement membrane material, in addition to being found within the somite itself. The term basement membrane material (BMM) will be used here to denote extracellular ground substance associated with a definite tissue interface, while the term extracellular matrix material (EMM) will be used to denote ground substance not associated with a tissue interface.

A. Basement membrane material

The basement membrane materials associated with the ectodermal, endodermal, neural and notochordal faces of the developing somite all show the same staining properties with the alcian blue–pH 0.5 and CEC–alcian blue methods.

These BMMs stain with pH 0.5 alcian blue and have a CEC between 0.4 M and 0.6 M MgCl₂ in the stage 7–15 embryo, with all staining abolished by methanol/HCl. These staining reactions indicate the presence of sulphated GAG of the chondroitin sulphate type (Fig. 1).

Digestion with testicular hyaluronidase results in the abolition of most of the staining, but a minor resistant fraction is visible in the BMM even after 24 h incubation with 1 mg/ml testicular hyaluronidase (Fig. 8). This resistant fraction has a CEC between 0.4 M and 0.6 M MgCl₂, indicating chondroitin sulphate B.

As the embryo develops, the staining intensity of BMM increases, reaching a maximum at about stage 18–19. The BMMs of these older embryos continue to show the staining characteristics previously described. At the peak of alcian blue staining intensity, however, a very small amount of stain is retained by the BMM when stained in 0.8 M MgCl₂. This sulphated GAG is hyaluronidase-resistant, but comprises only a minor part of the hyaluronidase-resistant fraction, most of which has a CEC between 0.4 and 0.6 M MgCl₂. These staining reactions indicate that in addition to chondroitin sulphate B, very small amounts of a more highly sulphated hyaluronidase-resistant GAG, possibly keratan sulphate, are present in the BMM.
The presence of a hyaluronidase-resistant fraction in these BMMs was confirmed by the metachromatic reaction with azure A in pH 1.5 glycine buffer (Szirmai, 1963) after hyaluronidase digestion.

The precise locations of these BMMs are as follows.

(1) **Ectodermal and endodermal BMM**

BMM is first found in association with the epiblast/mesoderm boundary in the primitive streak (stage 4) embryo. This BMM increases in alcian blue–pH 0.5 staining intensity as the ectoderm differentiates. In slightly older embryos BMM is also found at the hypoblast/mesoderm boundary, becoming the endodermal BMM. By the time that the first somites are delimited from the paraxial mesoderm, their ectodermal and endodermal surfaces are bounded by well developed BMM. The amount and staining intensity of the ectodermal and endodermal BMMs associated with somite mesoderm increase markedly between stages 12 and 18 (2.5–3.5 days).

In later stages this epithelial BMM is seen between the neural tube and ectoderm (Fig. 3) and between the notochord and endoderm where no mesoderm intervenes between the tissues.

(2) **Neural BMM**

The neural plate being continuous with the ectoderm in the young embryo, both are bounded by a continuous basement membrane. As neurulation proceeds and the neural tube is formed, BMM is deposited around its entire circumference.

(3) **Notochord sheath material**

The notochord becomes enveloped in BMM as it develops. This material is usually referred to as the notochord sheath. In early stages this sheath is relatively inconspicuous, with the notochord closely apposed to both neural and endodermal surfaces (Fig. 4). Later (stages 12–18) the sheath material increases both in amount and in staining intensity (Fig. 5), extending some distance from the surface of the notochord in an attenuated form.

Extracellular material with the same staining properties as the sheath can be detected within the notochord from stage 15 onwards, establishing the ability of the notochord cells to synthesize sulphated GAG-containing matrix material at this time. By stage 26 considerable amounts of intranotochordal matrix material are present (Fig. 11).

(4) **Nephrotome BMM**

This BMM delimits the developing nephric tubule from both the overlying ectoderm and from the underlying intermediate mesoderm (Fig. 6), and is detectable in stage 12 embryos.
NOTE. All sections stained with alcian blue at pH 0.5 and photographed with a Wratten 25 red filter.

Fig. 1. T.S. Posterior somite region of stage 16 embryo. Note basement membrane material surrounding the somite (so).

Fig. 2. T.S. Anterior-medial somite region of stage 16 embryo. Note extracellular matrix material accumulating within the sclerotome (s).

Fig. 3. T.S. Neural fold at stage 9. Note BMM between ectoderm and neural tube (arrow).

Fig. 4. T.S. Posterior notochord (n) at stage 9 with surrounding BMM.

Fig. 5. T.S. Anterior somite region at stage 16. Note attenuated material extending away from notochord surface towards the somite sclerotome (s).

Fig. 6. T.S. Medial somite region at stage 14. Mesodermal BMM between nephric tubule (nt) and intermediate mesoderm (im).
The extracellular matrix material (EMM) associated with the interior of the somites may be divided into two categories with respect to staining properties. Precartilaginous EMM has the same staining characteristics as basement membrane material, whereas cartilage matrix differs from precartilaginous EMM and BMM in the staining properties of its sulphated GAGs.

(1) Precartilaginous matrix material

Sulphated GAGs were not detected in somites of stages 7–13. Somites of stage 14, however, show small amounts of EMM between the cells of the somite ‘core’ (Fig. 7). This EMM has the same staining properties as the BMMs already described; that is, it stains with alcian blue–pH 0.5, has a major hyaluronidase-sensitive fraction with a CEC between 0.4 M and 0.6 M MgCl₂, and a minor hyaluronidase-resistant fraction, also with a CEC between 0.4 M and 0.6 M MgCl₂. These staining reactions indicate a major chondroitin sulphate A/C fraction and a minor chondroitin sulphate B fraction.

As the core cells disperse during the differentiation of the somite into sclerotome and dermomyotome, this EMM becomes temporarily undetectable (Fig. 1). Soon thereafter, however, it is apparent in thick (30 μm) sections that EMM is being deposited around the cells of the sclerotome, visible as fine wisps of material staining for sulphated GAGs (Fig. 2). As the sclerotome becomes distinct from the dermomyotome, the inner border of the epithelioid dermomyotome is particularly distinguished by deposition of EMM (Fig. 9).

The interior of the dermomyotome is devoid of EMM, but as the dermatome becomes distinct from the myotome EMM can be seen to permeate the dermatome, but not the myotome (Fig. 10). This sclerotomal and dermatomal EMM is continuous with similar material found in mesodermal connective tissues derived from lateral mesoderm. Epithelial, neural and endodermal tissues show no such EMM between the cells.

The staining properties of this mesodermal connective tissue EMM continues to match those of the developing BMM, with very small amounts of hyaluronidase-resistant sulphated GAG with a CEC in excess of 0.8 M MgCl₂ being detectable after stages 17–18.

(2) Cartilage matrix

Cartilage matrix is first detectable in the anterior somites of stage 21 between the cells of the sclerotome which have migrated around the notochord. Connective tissue EMM with a hyaluronidase-resistant fraction is found in the intervertebral regions of the somites, but between these regions the sclerotome cells are gradually separated from one another by the deposition of matrix material lacking a hyaluronidase-resistant fraction. This hyaluronidase-labile matrix material increases in staining intensity as these regions gradually become
NOTE. All sections stained with alcian blue at pH 0.5 and photographed with a Wratten 25 red filter.

Fig. 7. T.S. Posterior-medial somite of stage 14 embryo. Note EMM (arrow) between somite 'core' (co) but not 'cortex' (cx) cells.

Fig. 8. T.S. Posterior somite region at stage 12 after hyaluronidase digestion. Note residual staining of neural basement membrane (arrow) surrounding spinal cord (sc).

Fig. 9. T.S. Anterior-medial somite at stage 16. Note EMM staining (arrow) on inner boundary of dermomyotome (dm) and sclerotome (s).

Fig. 10. T.S. Anterior somite region at stage 18. 30 μm section. Note EMM staining in sclerotome (s) and dermatome (d) but not in myotome (m).

Fig. 11. T.S. Anterior region of stage 27 embryo. 30 μm section. Note intense staining of newly formed cartilage matrix (arrows) plus less intense staining of connective tissue EMM and BMMs.

Fig. 12. T.S. Anterior region of stage 27 embryo after hyaluronidase digestion. 30 μm section. Note complete absence of staining in cartilage matrix (arrow), while stain is still retained by BMM, notochord sheath and intranotochordal EMM.
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recognizable as histotypically differentiated cartilage. In spite of the intensity of staining for sulphated GAGs in developing cartilage, the matrix material remains completely labile to hyaluronidase up to stage 30. This distinction between the partially hyaluronidase-resistant BMM and connective tissue EMM and the labile cartilage matrix is seen in Figs. 11 and 12. The cartilage matrix sulphated GAG has a CEC between 0·4 M and 0·6 M MgCl₂ up to stage 30 and thus is probably composed entirely of chondroitin sulphate A/C.

Much later in the maturation of vertebral cartilage (after stage 33), small amounts of hyaluronidase-resistant sulphated GAG are demonstrable in the perichordal region and later also in the subperichondrial matrix. The greater part of this hyaluronidase-resistant material has a CEC in excess of 0·8 M MgCl₂, and thus probably represents keratan sulphate.

DISCUSSION

It is evident from the results obtained with alcian blue methods employed in the present study that the chick embryo is already synthesizing histochemically detectable sulphated glycosaminoglycans of the chondroitin sulphate type in the form of basement membrane material at the primitive streak stage (stage 4). No histochemically visible sulphated GAG was detected in association with the epiblast or hypoblast prior to the advent of mesodermal immigration at gastrulation, although Low (1967, 1968) has demonstrated by electron microscopy the presence of primary microfibrillar material along the basal surface of the epiblast of the newly laid egg blastodisc. Nevertheless, detectable sulphated glycosaminoglycan associated with basement membranes is detectable with alcian blue well before the 3rd day of incubation when it has been noted by O'Connell & Low (1970).

The interpretation of histochemical results obtained with alcian blue in terms of glycosaminoglycan biochemistry has shown to be valid when results of staining have been compared with biochemical microanalysis (Stockwell & Scott, 1967). Nevertheless it must be borne in mind that the CEC varies with both the sulphate ester content of the polyanion and with molecular weight (Scott & Stockwell, 1967). Thus it is possible for a low-molecular-weight keratan sulphate to have a CEC of less than 0·7 M MgCl₂ and thus to present the same staining pattern as chondroitin sulphate B. However, bearing these caveats in mind, the alcian blue–low pH and CEC methods do afford a means of distinguishing different categories of sulphated GAG even though the biochemical identification of the polyanion responsible for staining must be to some extent tentative.

Although the origin of the basement membranes associated with the tissue interfaces cannot be directly inferred from their location, the presence of such material between the ectoderm/neural tube, notochord/neural tube and notochord/endoderm indicates that in these locations the mesoderm is not involved in BMM synthesis. There is evidence from a variety of locations that both the
protein and sulphated GAG components of epithelial basement membranes can be synthesized by the epithelium (Pierce, 1966; Nadol & Gibbins, 1970; Dodson & Hay, 1971). Isolated 2-day neural tubes of the chick embryo have also been shown to be capable of synthesizing neural basement membrane material (Cohen & Hay, 1971). The BMM seen in association with somite and pre-somite mesoderm is therefore probably of ectodermal rather than mesodermal origin. The first histochemically detectable sulphated GAG undoubtedly synthesized by somite mesoderm is represented by the EMM observed within the core of stage 14 somites, although Manasek (1970) has reported uptake of $^{35}$S in mesoderm of stage 7 embryos which may represent sulphated GAG synthesis.

The marked deposition of BMM between the ectoderm and subadjacent somite mesoderm observed in the stage 12–18 embryo (2.5–3.5 days) may be related to the activity of this ectoderm in promoting somite chondrogenesis in chorioallantoic grafts with stage 9–11 somites (O'Hare, 1972).

McConnachie & Ford (1966) examining skate embryos with metachromatic staining methods also found a hyaluronidase-resistant fraction in dermal connective tissue and the notochord sheath which they identified as chondroitin sulphate B. In the present study the histochemical characteristics of the BMM and EMM are consistent with an unvarying sulphated GAG composition of CS-A/C and CS-B. The appearance of a detectable keratan sulphate-like material in later stages is probably accounted for by the overall increase in staining intensity of BMM and EMM that occurs, rendering a minor component visible as staining intensity increases. The failure to demonstrate a hyaluronidase-resistant GAG component in young cartilage matrix cannot be ascribed to this effect as intense hyaluronidase-labile staining may be apparent without a resistant fraction being demonstrable.

In qualitative terms it is clear that there is a distinct difference between the basement membrane and non-cartilaginous extracellular matrix sulphated GAGs on the one hand and those of newly formed cartilage matrix on the other. The sulphated GAGs synthesized by the newly forming cartilage are not identical with those found in pre-cartilaginous extracellular matrix material synthesized by somite mesoderm. The fact that cartilage matrix does differ qualitatively from the pre-cartilaginous matrix may explain why the pattern of incorporation of label from $^{14}$C-glucosamine into precursors of sulphated GAG by 2.5 to 3.5-day chick embryo somites differed from that shown by mature cartilage (Marzullo & Lash, 1967). It cannot therefore be held that glycosaminoglycan synthesis by pre-cartilaginous somite mesoderm represents a low level of cartilage matrix synthesis without recognizing that a qualitative as well as quantitative change occurs with the onset of overt chondrification.
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This investigation has been supported by grants to the Chester Beatty Research Institute (Institute of Cancer Research: Royal Cancer Hospital) from the Medical Research Council and the Cancer Campaign for Research.

REFERENCES


(Manuscript received 20 April 1972)