ACID MUCOPOLYSACCHARIDES IN THE DEVELOPMENT
OF THE PACIFIC GREAT SKATE, RAJA BINOCULATA

by

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ABSTRACT

Histochemical treatments specific for hyaluronic acid, chondroitin sulphate A/C, chondroitin sulphate B, and heparin, which are biological compounds classed as acid mucopolysaccharides, were applied to a series of Pacific great skate (Raja binoculata) embryos in order to characterize histochemically the acid mucopolysaccharides present in the embryos and to study the events leading to the situation of acid mucopolysaccharides localization in the adult. Embryonic stages examined ranged from early cleavage to immediate prehatching.

A progression was observed from; 1. intracellular neutral polysaccharides in cleaving stages through, 2. a combination of extracellular neutral polysaccharides and weakly acidic acid mucopolysaccharides (hyaluronic acid) associated with cell processes in neurulating stages to, 3. extracellular strongly acidic sulphated acid mucopolysaccharides (chondroitin sulphates) in later stages, particularly in areas of cartilage development.

In neurulating embryos hyaluronic acid appeared in considerable quantity between some adjacent tissue layers in a smooth layer form suggestive of some developmental significance for this compound. Hyaluronic acid also occurred in a similar form in lesser quantity in post neurulae (17-18 mm. embryos) in close association with

developing gut and mesonephros.

Results of histological tests in immediate prehatching embryos agreed with previously reported biochemical analyses of shark skins and cartilages i.e. chondroitin sulphate B occurred primarily in the skin and chondroitin sulphate A/C were a major component of the cartilage matrix.

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INTRODUCTION

Existing histochemical techniques permit the identification of acid mucopolysaccharides in tissue sections and considerable information is available on the acid mucopolysaccharide (AMPS) constitution of specific tissues in adult organisms. However, it has not yet been shown that AMPS can be characterized specifically in embryos and it has not yet been shown that the chain of events leading to the situation of AMPS localization in adults can be ascertained histochemically during embryonic development.

Previous observations in this laboratory of embryo Elasmobranch yolk stalks have indicated that sufficient AMPS are present to permit such a study (McConnachie and Ford, 1964). Accordingly, the problem selected was to investigate by histochemical means the AMPS occurring throughout the embryonic development of the Elasmobranch, Raja binoculata, the Pacific (great) skate.

Before describing the methods used to approach this problem it is necessary to discuss some of the

(Abbreviations used. AMPS- acid mucopolysaccharides, ABPAS- combined Alcian-blue-periodic acid Schiff stain for acid mucopolysaccharides, HA- Hyaluronic acid, C-S-A - Chondroitin sulphate A, C-S-B - Chondroitin sulphate B, C-S-C - Chondroitin sulphate C, CPB- cetyl pyridinium bromide)

available information on the nature of AMPS, their occurrence, and the techniques which have been used in their identification.

Mucopolysaccharides were first defined in 1938 as polysaccharides containing hexosamine occurring free or as esters of sulphuric acid. Acid mucopolysaccharides, in addition to hexosamine, have hexuronic acid as a second component and occur free or as sulphate esters. Compounds which are included in the acid mucopolysaccharide group are hyaluronic acid; the chondroitin sulphates A, B and C; keratosulphate and heparin.

Hyaluronic acid is a polymer of N-acetyl-D-glucosamine and D-glucuronic acid units which occurs as a free disaccharide in synovial fluid, Wharton's jelly and vitreous humours and is a component of cartilage matrix. (Pearse, 1961; Shaw and Martin, 1962; Dorfman, 1963).

The chondroitin sulphates are one of the main acid mucopolysaccharide components of connective tissues and particularly of cartilage matrix. In mammalian cartilage, chondroitin sulphates A and C may account for up to 40% of the dry weight, (Bloom and Fawcett, 1962) and are thought to occur in covalent linkage with cartilage proteins (Dorfman, 1963). Chondroitin sulphate B occurs in aortas and is the major sulphated AMPS of skin (Dorfman, 1963), C-S-A and C are polymers of N-acetyl-D-galactosamine and D-glucuronic acid units, differing one from another only

in the position of their sulphate groups. C-S-B is a polymer of N-acetyl-D-galactosamine and D-iduronic acid. It is not labile to the action of testicular hyaluronidase as are C-S-A and C (Walker, 1961).

Keratosulphate, first isolated from cornea, is now known to occur in cartilage. With increasing age it replaces the chondroitin sulphates in human costal cartilage and in rabbit nucleus pulposus (Dorfman, 1963; Davidson and Small, 1963). Keratosulphate is a polymer of equal amounts of N-acetyl-D-glucosamine, D-galactose and sulphate. Little is known of the glycosidic linkages and response to hyaluronidase of keratosulphate.

Heparin, unlike the extracellular connective tissue AMPS, is cellular and is found associated with strongly basic protein in mast cells (Spicer, 1963; Schiller, 1963). Heparin is a polymer of D-glucosamine and D-glucuronic acid with 3 sulphates per dissaccharide unit (Walker, 1961).

Structural and chemical data pertaining to the above mentioned AMPS are presented in Appendix A.

Recent investigations have shown that AMPS have a wide distribution in different animal classes and tissues (Anno, Seno and Kawaguchi, 1962; Szabo and Roboz-Einstein, 1963; Kato and Sirlin, 1963; Mathews, 1962; Spicer, 1960 and 1963; Zugibe, 1963). To date, only a few investigations have examined the synthesis of AMPS in embryonic material. Such studies have used isotope

incorporation and enzyme-assay techniques with cell systems (Ozello and Bambray, 1964; Prockop, 1964; Schiller, 1963; Spicer, 1963).

New developments in histochemical techniques make it possible to differentiate between some AMPS in tissue sections, and the use of enzyme extractions and specific blocking techniques make further, more sophisticated differentiation possible.

Kelly, Bloom and Scott (1963) showed that AMPS behaved histochemically as complex polyanions which could be precipitated by quaternary ammonium detergents and selectively unblocked according to their dissociation at different molar concentrations of inorganic electrolytes.

Saunders (1964) developed a fluorescent staining method for AMPS using Acridine orange at varying pH levels. This allows differentiation of hyaluronic acid, the chondroitin sulphates, and heparin in tissue sections through a blocking and unblocking technique similar to that of Kelly, Bloom and Scott.

Szirmai (1963) suggested that cationic dyes such as Azure A would form metachromatic precipitates with AMPS in a 1:1 ratio between anionic sites on disaccharide units and dye molecules. The electrostatic nature of dye-binding means that it will depend upon the pH of the medium and the pK values of the anionic groups involved. Thus hyaluronic acid containing only one carboxyl group per disaccharide unit will bind one dye molecule at neutral pH.

Lowering the pH will decrease the carboxyl group dissociation and below pH 2 dye-binding is extinguished. Chondroitin sulphate in contrast, contains both a carboxyl and a sulphate group. At pH 2, the sulphate group is still strongly dissociated and the disaccharide will bind one dye molecule. On this basis, Azure A at pH 1-2 binds to sulphate groups.

Yamada (1963b, 1964) studied the staining reactions of artificial and natural sulphated polysaccharides with Alcian blue, Alcian blue-PAS and Azure A stains using blocking techniques of methylation-demethylation and acetylation-deacetylation. He suggested that Alcian blue staining of AMPS is not necessarily dependent on the acid groups in them and that it might be advisable to examine their acidity by other staining properties such as thiazine dye metachromasia at different pH levels.

The studies of Spicer (1960, 1963) concerned the great variety of AMPS in rodent epithelial mucins and made a comparison of mast cell staining in different rodent species. He used ABPAS, Azure A at different pH levels, methylation-demethylation, and sulphation treatments. He found so great a diversity of AMPS staining responses that classification was difficult. He suggested a classification of AMPS based on relative acidity, azurophilia and alcianophilia. He also found that mast cells are strongly azurophilic even at pH 0.5 and are non-reactive with Alcian blue. This lack of alcianophilia coupled with strong azurophilia is characteristic of highly sulphated AMPS.

Enzyme extractions have not been widely used for the identification of AMPS. Spicer (1960) mentioned hyaluronidase as a possible means of aiding histochemical identification of AMPS but considered it nonspecific. However, the action of testis hyaluronidase is quite specific, hydrolyzing hyaluronic acid, C-S-A and C-S-C (Pearse, 1961; Walker, 1961; Zugibe, 1961). Malt diastase* and amylases have been used for the removal of glycogen (20 minutes at room temperature, 1% solution in distilled water) and, particularly, before sulphation treatments, to prevent sulphation of glycogen (Yamada, 1962). Malt diastase contains many fractions however, and if incubation is prolonged, cartilage matrix metachromacy and cytoplasmic basophilia will be removed (Pearse, 1961). Thus it is possible that a sequence of enzyme treatments and inorganic electrolyte extractions coupled with staining treatments which depend on relative acidity could provide definite information on specific AMPS identification and localization.

* Analytical malt diastase, National Biochemical Corporation.

MATERIALS

In this study of AMPS development, embryos of the elasmobranch, Raja binocularata, were used, since, as it is a cartilaginous fish, a high concentration of AMPS could be anticipated and it is readily available in egg cases from January through September.

The biochemical investigations of Seno and Meyer (1963) have demonstrated the presence of hyaluronic acid and C-S-B in shark skin of two species as well as the presence of C-S-C in skeletal cartilage. Further works (Mathews, 1962) have shown the presence of C-S-C, protein and sulphated keratosulphate in cartilages from Squalus acanthis and the sting ray Dasaytis and, in a comparative study of whale, bovine, shark and squid cartilages (Anno, Seno and Kawaguchi, 1962) the presence of both glucosamine and galactosamine fractions indicated a constant involvement of chondroitin sulphates and a less predictable involvement of hyaluronic acid in the cartilages studied.

On this basis it was anticipated that AMPS development in embryos of Raja binocularata would lead to the presence of hyaluronic acid and C-S-B in skin and C-S-C and possibly hyaluronic acid in cartilage in the adult stages.

Egg cases were obtained by trawl off Tsawassen in the Straits of Georgia at 16 fathoms and were maintained in sea water at 12°C. A large stock was held because the

developmental stage could not be determined from the external appearance of the case and many cases were empty. The incubation times of rays ranges from 9-15 months but is not known for Raja binocularata and may be in excess of 15 months. (Tewinkel, 1960, 1963; Libby and Gilbert, 1960; Clark, 1919) The rate of development of R. binocularata is slow and there is no difficulty in obtaining recently fertilized eggs and a succession of developmental stages.

METHODS

Fixation

Acid mucopolysaccharides (AMPS) require special fixation because they occur in tissues in a highly hydrated state and because of their ionic nature. (See Appendix A)

Formalin is used frequently (Yamada, 1963; Spicer, 1960, 1963; Lhotka, 1964) but is probably effective only in precipitating protein and any AMPS in combination with that protein (Pearse, 1961; Zugibe, 1963; Szirmai, 1963). Both Zugibe and Szirmai stated that alcoholic and acidic fixation of AMPS were not recommended because the former is reversible in aqueous solutions and the latter may change the ionic nature of AMPS. Both authors suggested that large cations e.g. cetylpyridinium chloride or bromide (CPC or CPB) in combination with formalin would improve AMPS fixation as both protein bound AMPS and free AMPS would be precipitated. However, such ionic precipitation blocks anionic groups of AMPS. If staining is to involve these anionic groups, an acidic staining medium is required to break the salt linkage.

Kelly, Bloom and Scott (1963) compared a number of fixatives used to precipitate AMPS and found CPC-formalin to be the best fixative for both nucleic acid and AMPS. Morphological detail was comparable to that obtained with neutral formalin and large amounts of amorphous ground

tissue substance were retained.

Accordingly, the fixative selected was CPB-formalin as described by Williams and Jackson (1956) and modified by Kelly, Bloom and Scott (1963).

Staining

AMPS possess a number of binding sites available for combination with cationic dyes such as Azure A. These are carboxyl, sulphate and, under certain conditions, hydroxyl groups. Cationic dyes will bind one dye molecule at any free anionic site. Which sites are available is determined by the pH of the staining medium. Thus, in Azure A at pH 4.0 both carboxyl and sulphate groups are ionized and free and dye binding can occur at both sites. With Azure A at pH 1.5 only sulphate groups are ionized and dye binding is decreased considerably or not depending on the proportion of carboxyl and sulphate groups (Zugibe, 1963; Szirmai, 1963; Spicer, 1963).

Many cationic dyes are members of the thiazine group e.g. Azure A, Toluidine blue, Gresyl violet, in which the phenomenon of metachromasia occurs. This is a polymerization of dye molecules depending on a number of factors, the most important being surface density of negative charges on the dye substrate (Pearse, 1961). Hyaluronic acid, according to Pearse, shows no metachromasia

in dilute aqueous solutions because the distance between adjacent carboxyl groups in the polysaccharide chain is about $10.3 \text{ \AA}$ which is not sufficiently close for dye-stacking to occur. Polysaccharides containing alternate sulphate and carboxyl groups i.e. Chondroitin sulphate, would have an interchange distance of 4 and $6 \text{ \AA}$ and some moderate, stable metachromasia would be expected.

Two states of metachromasia can be recognized, the beta or violet form and the red or gamma form. The beta (violet) form is due to the simultaneous presence of the alpha (orthochromatic) form and the gamma form. However, for the purpose of this study, significance was attached only to the presence or absence and the relative intensity of metachromasia.

Sections were stained with Azure A at pH 1.5 or 4.0 to examine their relative acidity due to carboxyl or sulphate groups. Alternately, sections were stained by the Alcian blue-PAS reaction of Lison as adapted by Spicer (1960). AMPS are shown by Alcian blue staining at pH 2.7 and neutral polysaccharides by the red or pink PAS staining. A magenta (blue-red to purple) reaction indicates staining of acidic polysaccharides. (See Appendix B for stain procedures.)

Blocking reactions were used in conjunction with both Azure A and Alcian blue-PAS (ABPAS) techniques.

The methylation and demethylation sequence blocks the staining of acid groups by hydrolyzing sulphate esters

and forming methyl esters on carboxyl groups. Demethylation hydrolyzes methyl esters and thus restores carboxyl groups only (Yamada, 1963b).

Acetylation and deacetylation blocks amino and hydroxyl group staining and restores hydroxyl staining only (Yamada, 1963b). This technique is more useful in conjunction with ABPAS staining which involves hydroxyl groups in particular.

Sulphation is used to produce ester sulphate groups and the results are generally interpreted as esterifications of carbohydrate hydroxyl groups (Yamada, 1962).

Enzyme extractions were used with both staining treatments; malt diastase to remove glycogen and, with prolonged incubation, for partial polysaccharide degradation (Pearse, 1961; Culling, 1963); hyaluronidase to remove Hyaluronic acid, C-S-A and C-S-C (Pearse, 1961; Shackleford and Bentley, 1964).

On some sections differential salt extractions were substituted for enzyme extractions. Hyaluronic acid is removed in 0.3 M NaCl and both hyaluronic acid and the chondroitin sulphates are removed in 0.6 M NaCl. These extractions were carried out according to the method of Kelly, Bloom and Scott.

On some sections, particularly in older embryos, Saunders' (1964) Acridine orange technique was used for identification of hyaluronic acid, the chondroitin sulphates and heparin in parallel sections.

All of the preceding techniques are described in Appendix B.

The preceding treatments were applied to embryos of the following developmental stages:

- (a) Cleavage stages, blastulae and gastrulae.
- (b) 2-4 mm. head fold embryo, short foregut, early neurula.
- (c) 6-7 mm. embryo, neurulating stage, first head torsion in progress, foregut and hindgut formed, somitic, splanchnic and hypomeric mesoderm apparent in midgut area.
- (d) 17-18 mm. embryo, blood islands forming in extraembryonic mesoderm, double head torsion complete, first spontaneous movements apparent in tail, intestine and mesonephros forming.
- (e) 4 cm. embryo, branchial filaments 1.5 cm. in length, out growth of pectoral fins in progress, extraembryonic circulation complete.
- (f) 7 cm. embryo, branchial filaments being resorbed, width of pectoral fins 1.4-1.6 cm., still increasing, ocelli not present on pectoral fins but pigmentation of epidermis appearing, sexual dimorphism apparent but anal membrane still present.
- (g) 14 cm. embryo, pectoral width 5.5 cm., no external branchial filaments, ocelli present, anal membrane split, last prehatching stage.

Embryos were removed from their eggs, fixed in CPB-formalin for 48 hours at room temperature, dehydrated through ethanol, cleared in xylene, paraffin embedded, sectioned at 8 microns and examined by the techniques described.

Between 2 and 7 embryos of each developmental stage were examined. Frequently, parallel series of sections were made from one specimen so that adjacent sections from an embryonic region could be subjected to several treatments. This was useful in developmental stages which had only 2 or 3 available embryos.

RESULTS

Cleavage Stage

The cleavage stage embryos show very little azurophilia and in contrast, considerable PAS reactivity. Details of results are shown in Table I. The lability of the slight Azure A staining at pH 4 to hyaluronidase digestion suggests the presence of a slight amount of hyaluronic acid and this is supported by the absence of staining at pH 1.5 which shows an absence of strongly acidic AMPS.

Sulphation increased azurophilia 3 to 5 fold even in yolk which does not normally stain. If sulphate esters are formed on free hydroxyl groups, this indicates the presence of a high density of hydroxyl groups which are possibly on oligosaccharides (neutral polysaccharides).

ABPAS staining was largely due to PAS reactivity although some Alcian blue staining was present to account for the magenta rather than pink coloration. Diastase incubation reduced alcian-ophilia but did not affect PAS reactivity, further supporting the presence of neutral polysaccharides. Acetylation abolished all staining with the exception of some slight alcianophilia in ventral portions of cells and saponification restored all PAS reactivity. On this basis it is apparent that almost all staining is due to hydroxyl groups of neutral polysaccharides

TABLE I

CLEAVAGE STAGES - BLASTULA TO GASTRULA
(No. of embryos 4)

TREATMENT	Superficial portions of cells	Deep portions of cells	Cleavage furrows	Cytoplasm adjacent to furrow	Yolk	Disc gradient <u>Uppermost</u> <u>Lowermost</u>
Azure A pH 4.0	O $\pm$	O+	O++	O+	-	$\frac{O^+}{O^\pm}$
Azure A pH 1.5	-	-	-	-	-	-
Hyalase A.A pH 4.0	-	-	-	-	-	-
Sulphate A.A pH 4.0	O+++	O+++	O+++	O+++	O+++++	-
ABPAS	AB+	ABP+++	ABP+++	ABP++++	PAS+	$\frac{AB+}{ABP+++}$
Diastase ABPAS	PAS+	PAS+++	PAS++++	PAS++++	PAS+	$\frac{+}{+++}$
Acetylate ABPAS	-	AB $\pm$	-	-	-	$\frac{-}{AB\pm}$
Acetylate Saponify ABPAS	PAS++	PAS+++	PAS++++	PAS++++	PAS++	$\frac{++}{+++}$

O = an orthochromatic reaction; AB = Alcian blue reaction;
PAS = Schiff reaction; ABP = concurrent Alcain Blue and Schiff
reaction; - = negative reaction; + = positive reaction the
number of +'s being proportional to the intensity; $\pm$ = doubtful
reaction.

as the acetylation-saponification treatment blocks and restores hydroxyl groups. This means that the slight azurophilia is a reflection of the small amount of acidic materials present.

A gradient of PAS staining occurred which is suggestive of fixation polymerization of glycogen (Pearse, 1961). This is shown in Figure 1. Because the gradient persists after diastase treatment it can not be called glycogen polymerization but it may well be due to fixation. The greatest staining density is at the cell boundaries (membranes) and in the tissues adjacent to the lateral and ventral boundaries of cells. The deep portion of any cell is more heavily stained than the superficial half and the bottom part of the disc is more heavily stained than the top. In this connection, it was apparent in control slides that alcian blue staining alone occurred only in the uppermost surface of cells i.e. the top 0.025 mm.

All staining was cytoplasmic and very granular because the cytoplasm was so yolky. Few nuclei were observed and those that occurred were in mitotic figures about 20 microns in diameter.

2-4 mm. Embryo

Extracellular staining associated with mesenchyme cell processes appeared in the 2-4 mm. head fold embryos. This was designated as extracellular material

Figure 1. Cleavage stage, ABPAS showing PAS gradient, x 42.

(ECM) and was also found as a thin sheet or basement membrane like structure on the ventral surface of the neural ectoderm and epidermis. Thin strands of ECM extended from ectoderm to the underlying mesoderm (Figures 2, 3). Azure A results indicated that the ECM was a weakly acidic AMPS containing hydroxyl and carboxyl groups, labile to hyaluronidase, and present only in very limited amount (See Table II). This same type of AMPS was found in a thin sheet surrounding the notochord (perinotochordal material) and was recognized as possibly being a low concentration of hyaluronic acid. ABPAS results do not support this entirely, but do indicate that some carboxyl groups are involved in staining. This is shown in the methylation-demethylation sequence. ABPAS results also indicate that neutral polysaccharides are present in all tissues in higher concentration than any weakly acidic AMPS. This is shown by the acetylation blockade and deacetylation restoration of PAS staining. Polysaccharide (neutral) concentrations were most prevalent in epidermal cells and notochord cells, less so in endoderm cells and least in mesoderm cells.

6-7 mm. Embryo

In the 6-7 mm. embryo, the neural tube is complete. The hypomere has separated into somatic and

TABLE II

2-4 mm. HEAD FOLD EMBRYO
(No. of embryos 4)

TREATMENT	Ectoderm <u>dorsal</u> ventral	ECM	Mesoderm	<u>Perinot.</u> Notoch.	Endoderm	Yolk
Azure A pH 4.0	O++ O++	O+----	O+±	O+++ O+±	O+±	-
Azure A pH 1.5	-	-	-	-	-	-
Hyalase Azure A pH 4.0	-	-	-	-	-	-
Diastase Azure A pH 4.0	O± O±	O+----	O±	O+++ O±	O±	-
Methylate Azure A pH 4.0	-	O±	-	-	-	-
Methylate Saponify Azure A pH 4.0	O± O++	O±---	O±	O+ O±	O±	-
ABPAS	ABP++ PAS++++	ABP+++++	ABP++	ABP++ PAS++++	PAS+++	PAS+
Hyalase ABPAS	PAS+ PAS++++	AB±----	PAS++	PAS++ PAS+++	PAS+++	PAS+
Acetylate ABPAS	AB± AB±	-	AB±--	AB+ AB±	AB±	-
Acetylate Saponify ABPAS	PAS+ PAS+++	-	PAS++	PAS++ PAS+++	PAS++	PAS+
Methylate ABPAS	PAS± PAS++	AB++	PAS+	ABP++ PAS++	PAS++	PAS±
Methylate Saponify ABPAS	ABP+ ABP++	AB+++	PAS++	ABP+++ PAS+++	PAS+++	PAS+

Figure 2. 2-4 mm. embryo showing ECM of the cellular extension form. Azure A pH 4.0, x 200.

Figure 3. Portion of 2-4 mm. embryo showing smooth layer form of ECM (S) around notochord (N) and on ventral surface of neural ectoderm. ABPAS, x 340.

Figure 4. 6-7 mm. embryo showing extent of ECM (E). ABPAS, x 46.

Figure 5. High power view of mid-trunk portion of 6-7 mm. embryo showing smooth layer form of ECM (F) around notochord (N) and subnotochordal rod (S), and 'pseudopod' form of ECM (P) extending from subnotochordal rod to intestine (I) and lateral splanchnic mesoderm. Azure A pH 4.0, x 1230.

and splanchnic components over the length of the midgut. The ECM is much more extensive than in the 4 mm. stage and is found associated with cell processes between mesoderm and any adjacent tissue but not between somatic and splanchnic mesoderm. A sheath of ECM was present around the notochord (perinotochordal sheath) but was much thicker, about 1.2 microns, than in the 4 mm. stage where it was less than a half micron in thickness. Figure 4 and 5 show the embryonic form and extent of the ECM in the 6-7 mm. embryo.

Staining results clearly show that at this stage of development all extracellular material is entirely AMPS in nature, (Table III) and is in a state of higher polymerization than in earlier stages. This is shown by the first appearance of metachromasia in Azure A staining of ECM.

Azure A pH treatments indicate the presence of some strongly acidic AMPS as well as considerable weakly acidic AMPS. This is shown by weak azurophilia at pH 1.5 and by strong metachromasia at pH 4.0. It may be that hyaluronic acid is a major component of the ECM with some sulphated AMPS, probably C-S-A or C-S-C being a minor component. This is supported strongly by the methylation blockade of azurophilia indicating that acid groups are responsible for staining, and by the hyaluronidase extraction blocking all ECM staining which indicates that the sulphated AMPS is C-S-A or C-S-C. It is notable that

TABLE III

6-7 mm. EMBRYO AZURE A CHEMICAL AND ENZYME TREATMENTS
(No. of embryos 7)

TREATMENT	ECM	Ectoderm	Neural ectoderm	Mesoderm	<u>Perinot.</u> <u>notoch.</u>	<u>Syncytial</u> <u>Nuclei</u> <u>Endoderm</u>
Azure A pH 4.0	M++	O±	O±-++	O++	M-++ -	<u>O+++</u> O+
Azure A pH 1.5	O±	-	-	-	O± -	<u>-</u> -
Methylate A.A pH 4.0	-	-	-	-	-	-
Sulphate A.A pH 4.0	M++-+++	-	-	-	M++-+++ -	-
Sulphate A.A pH 1.5	O+-++	-	-	-	O±-+ -	-
Acetylate A.A pH 4.0	O+-M±	O±	O+	O+-++	O± -	<u>O++</u> O±-+
Acetylate Saponify A.A pH 4.0	-_O+++	-	-	-	-	<u>O++</u> -
Hyalase A.A pH 4.0	-_O++++*	-	-	-	-	-
Diastase A.A pH 4.0	O+++*M+	-	O±	O±	M+ -	<u>O±</u> O+
Hyalase Diastase A.A pH 4.0	-	-	-	-	-	-
R.N.Aase A.A pH 4.0	O+-M++	-	-	-	M+++ -	-

* Orthochromatic material is found lining anterior midgut lumen only.

TABLE III CONT.

ALCIAN BLUE PAS CHEMICAL AND ENZYME TREATMENTS AND SPECIAL TECHNIQUES

TREATMENT	ECM	Ectoderm	Neural ectoderm	Mesoderm	<u>Perinot.</u> <u>notoch.</u>	<u>Syncytial</u> <u>Nuclei</u> <u>Endoderm</u>
ABPAS	AB++++	ABP <u>±</u>	ABP++	ABP+	AB+++++ ABP <u>±</u>	ABP++ PAS++
Hyalase ABPAS	AB++	—	PAS+	PAS+	AB+++ PAS <u>±</u>	AB++ PAS++
Diastase ABPAS	AB++++	PAS <u>±</u>	PAS+	PAS <u>±</u>	AB++++ —	ABP++ PAS++
Hyalase Diastase ABPAS	AB+	PAS <u>±</u>	PAS <u>±</u>	PAS+	AB+++++ —	AB++ PAS++
R.N.Aase ABPAS	AB++++	PAS <u>±</u>	PAS+	PAS+	AB+++++ PAS <u>±</u>	AB++ PAS++
Sulphate ABPAS	AB++++	—	—	—	AB+++++ —	AB++++ —
Pyronin	++++	+	+	+	+++ +	++ +
R.N.Aase Pyronin	++++	<u>±</u>	<u>±</u>	<u>±</u>	+++ <u>±</u>	+ <u>±</u>
Saunders 1	++++	++	+++	+++	++++ +++	++ +
Saunders 2	—	—	+	+	<u>±</u> —	— —
Saunders 3	—	—	—	—	— —	— —

Saunders 1 stains for hyaluronic acid.

Saunders 2 stains for chondroitin sulphates and heparin.

Saunders 3 stains for heparin.

a strong orthochromatic reaction lining the lumen of the anterior midgut persists after hyaluronidase extraction and after diastase extraction, but not after a consecutive hyaluronidase and diastase extraction. (Figure 6) That this material is not AMPS in nature is inferred by its absence in Acridine orange and ABPAS staining. It may possibly be some form of bound lipid. The absence of PAS staining in ABPAS results show that both ECM and perinotochordal sheath contain little or no neutral polysaccharides. These results also suggest a difference between the two areas. A consecutive hyaluronidase and diastase extraction blocks or removes considerable ECM alcianophilia but has no effect on perinotochordal sheath alcianophilia. Azure A results do not suggest any difference as both regions respond to treatments in the same manner but the Saunders Acridine orange results indicate that sheath material contains some chondroitin sulphates and the ECM does not. On the other hand, Azure A results at pH 1.5 suggest that both areas contain similar proportions of chondroitin sulphates, which on the basis of hyaluronidase extractions are probably C-S-A and/or C-S-C. It is suggested that the sheath material contains demonstrable C-S-A and/or C-S-C which is not yet present in ECM in sufficient concentration to stain with Acridine orange.

Cytoplasmic staining in the 6-7 mm. embryos is not as dense as in the 2-4 mm. stage in ABPAS treatments and is about equal in Azure A treatments. With Azure A

Figure 6. AMPS like material (A) lining ventral portion of lumen of anterior midgut of 6-7 mm. embryo. Azure A pH 4.0 following hyaluronidase digestion, x 230.

all cytoplasmic staining is hyaluronidase labile, RNAase labile and partially labile to diastase. This is expected as cytoplasmic basophilia is partially due to RNA and RNA-protein. There is variation in amount of cytoplasmic azurophilia among tissues, as the neural tube and hypomeric mesoderm are intensely stained, epidermis and endoderm are weakly stained and notochord cells do not stain. ABPAS results show some persistent PAS reactivity which is moderate in endoderm, weak in neural tube, very weak in hypomeric mesoderm and epidermis and absent in notochord cells. Cytoplasmic alcianophilia is in a similar pattern to cytoplasmic azurophilia but does not occur in endoderm.

The Saunders reaction for hyaluronic acid indicates that neural ectoderm, mesoderm and notochord cells contain similar amounts of hyaluronic acid, epidermal cells contain less and endoderm cells contain least, which is in general agreement with the preceding results.

The syncytial nuclei found in the yolk syncytium of the midgut contain hyaluronic acid according to the Saunders and ABPAS treatments. However, their azurophilia is abolished by RNAase extraction and sulphation. It is more likely that this azurophilia is due to some acidic material other than AMPS or nucleoprotein or nucleic acid as sulphation abolishes cytoplasmic staining but does not affect nuclear azurophilia (Figure 7).

The pyronin treatments were included for this

Figure 7. Portion of trunk of 6-7 mm. embryo showing absence of cytoplasmic staining with Azure A pH 4.0 following sulphation, x 330.

developmental stage only as a check on possible RNA or RNA-protein participation in ECM staining. It was observed previously in this laboratory that methyl green-pyronin staining for DNA and RNA showed extensive pyroninophilia in ground substance and basement membrane structures, and this stain is also used for immunologically active mast cells where pyronin stains AMPS-like substances (Chen, 1963). Results of this treatment before and after RNAase incubation showed that RNA or RNA-protein was involved in cytoplasmic pyroninophilia fairly extensively and in syncytial nuclear pyroninophilia to a lesser degree but not at all in ECM and perinotochordal sheath staining (Figure 8).

In summary, in the 6-7 mm. embryo, hyaluronic acid appears to be a considerable part of ECM and perinotochordal sheath material, and C-S-A and/or C-S-C are a small part of sheath material and possibly part of the ECM but not yet definitely demonstrable. The cytoplasmic neutral polysaccharide content is much reduced in comparison to the 2-4 mm. embryo and the cytoplasmic content of hyaluronic acid is greater than the neutral polysaccharide content in mesoderm and neural ectoderm cells. AMPS content in epithelial ectoderm, notochord and endoderm cells appears to be negligible.

Figure 8. 6-7 mm. embryo showing ECM (E) staining with pyronin. Slide was incubated in RNAase and stained with methyl-green pyronin for nucleic acids. x 268.

17-18 mm. Embryo

In the 17-18 mm. embryo extracellular AMPS are not present in the form and extent shown in the 6-7 mm. embryo. The notochordal sheath is about the same thickness as in the 6-7 mm. stage although 2-3 times the diameter but ECM as such is no longer present except for a small amount between mesoderm and epidermis in the mid-trunk region. However, definite basement membrane like structures of AMPS content are present between endoderm and mesoderm of organ structures, e.g. heart and gut tube. In addition, there is a basement membrane involving mesoderm alone surrounding the developing mesonephric tubule.

AMPS are now indicated in quantity in mesenchyme cells which are in close proximity to the notochordal sheath and are packed loosely in comparison to the close packed trunk mesoderm (Figures 9, 10).

The notochordal sheath consists of 2 or 3 thin layers of matrix like material which is metachromatic at pH 4.0. At pH 1.5 there are 2 layers, one orthochromatic and the other slightly metachromatic. Both layers are labile to hyaluronidase extraction which indicates that staining at pH 1.5 is due to C-S-A/C (C-S-A and/or C-S-C). Some hyaluronic acid is possibly present to account for the intense staining at pH 4 but this could also indicate carboxyl group staining in the sulphated AMPS. The

Figure 9. 17-18 mm. embryo showing Hyaluronic acid in basement membrane form around mesonephric tubules (MT) and in fibroblasts (F) peripheral to notochord (N). ABPAS, x 240.

Figure 10. Hyaluronic acid in basement membrane form around gut tube rudiment (G). ABPAS, x 220. 17-18 mm. embryo.

acetylation-saponification treatments fail to suggest any neutral polysaccharide participation in staining and the methylation-saponification treatment may be taken as an indication of sulphate group staining. Results are given in Table IV.

The basement membrane like structures were metachromatic at pH 4, completely labile to hyaluronidase digestion and failed to stain at pH 1.5 which indicates that they are primarily hyaluronic acid. The response to the acetylation and methylation sequences is not the same as in the notochord sheath and only indicate that carboxyl groups may be responsible for some staining.

The presence of acidic and some neutral polysaccharides in notochord cells is inferred by the predominant PAS reaction, the methylation blocking and the saponification restoration of PAS staining.

Azure A staining suggests weakly acidic AMPS in the sheath mesenchyme cells by the metachromacy at pH 4, and by the absence of staining at pH 1.5 and, after hyaluronidase digestion. The resistance of alcianophilia to acetylation and the partial loss of alcianophilia following methylation support the suggestion and indicate that this weakly acidic material is hyaluronic acid.

Both trunk mesoderm and endoderm show less reactivity to Azure A and to ABPAS than the mesenchyme. The mesoderm stains more intensely than endoderm with Azure A and PAS which is the reverse of what occurred in

TABLE IV

17-18 mm. EMBRYO (No. of embryos 2)

TREATMENT	Notoch.	Notoch. sheath	Sheath mesenchyme	Trunk mesoderm	Trunk endoderm	Endo- mesod. base. membr.
Azure A pH 4.0	O+	M++	O+++-M++	O+++-M _±	O+	M+++
Azure A pH 1.5	-	M _± -O+	-	-	O+	-
Hyalase A.A pH 4.0	-	-	-	-	-	-
Acetylate A.A pH 4.0	-	M++	O++-M+	O++	O _±	M++
Acetylate Saponify A.A pH 4.0	O _±	M _±	-	O++	O++	-
ABPAS	PAS+++	ABP++++	AB++++	PAS++	PAS+	AB++++
Acetylate ABPAS	PAS+++	ABP++++	AB++++	PAS _±	PAS _±	AB++++
Acetylate Saponify ABPAS	PAS+++++	PAS++	AB++++	ABP++	ABP+	AB++++
Methylate ABPAS	PAS+	AB++	AB++	PAS _±	PAS+	AB+++
Methylate Saponify ABPAS	PAS++++	PAS++	PAS _±	PAS _±	PAS+	PAS+++

the 6-7 mm. embryos. This suggests some AMPS content in trunk mesoderm associated with the derivation of basement membrane structures. It is notable that in both trunk mesoderm and endoderm the acetylation-saponification sequence induced alcianophilia which was not present in control sections. This is in agreement with Yamada's (1963b) suggestion that alcian blue staining may involve groups other than carboxyl and sulphate units.

4 cm. Embryo

In the 4 cm. embryo, initial development of organ systems is well established. A definite Wolffian duct is present with 30-40 mesonephric tubules joining collecting tubules. Two large lobes of liver and a pancreatic rudiment are present. The oesophagus is still closed completely but the beginning of the spiral valve is visible in the intestine (Figure 11).

At this developmental stage, AMPS were restricted to the notochordal sheath, the associated sheath mesenchyme (fibroblasts), and trunk mesoderm. No basement membrane structures associated with trunk mesoderm displayed AMPS content. The structure of the sheath is complex; three definite layers may be demonstrated within it. (Figures 12, 12a) This complexity however is not present in the most anterior 2 mm. of the notochord. The anterior end of

Figure 11. General morphology of 4 cm. embryo.
Section through pectoral fins. ABPAS, x 170.

the notochord is well forward under the hindbrain at the same level as the epiphysis and it does not reach maximum diameter and complexity of structure until approximately 1.8 mm. posteriad (Table V). There is a distinctive area of peripheral mesenchyme cells with a matrix of AMPS which begins at 0.45 mm., reaches a maximum lateral extension from the notochord at 2.2 mm. (anterior end of the notochord is taken as 0) and by 4 mm. is minimal. The extension of this matrix is laterad and slightly dorsad toward the floor of the hindbrain. It reaches its greatest extent only after the notochord is adjacent to the floor of the hindbrain (Figures 13-16a). The AMPS material in this matrix is shown to be C-S-A/C and hyaluronic acid by both Azure A and Acridine orange staining. This suggests precartilaginous materials which are to be incorporated into the parachordal elements of the chondrocranium.

The three layered sheath structure is present 0.45 mm. from the anterior end of the notochord. The inner surface of the sheath is the outer layer of the notochord and is a squamous epithelium. This is densely stained with Azure A at pH 4 and slightly stained at pH 4 and 1.5 following hyaluronidase digestion. Diastase decreases Azure A staining markedly and reduces PAS staining significantly. Neutral polysaccharides are indicated to be the major constituent of this area although there may be a slight amount of AMPS as indicated by Acridine orange results (Table Va).

TABLE V

MEASUREMENTS OF ANTERIOR PORTIONS OF NOTOCHORD OF 4 cm. EMBRYO

Distance from Ant. End	Maximum Width	Maximum Height	Maximum extent of peripheral Mesenchyme AMPS (lateral)	Minimum Verticle dist. from Hindbrain Floor
0	44	44	-	275
150	55	77	-	175
250	66	83	-	110
350	100	122	-	55
450	150	110	28	39
650	165	150	38	11
1030	192	165	55	22
1200	220	148	110	38
1500	220	190	120	11
1800	275	202	165	0
2000	330	250	192	0
2200	300	275	250	0
2700	300	230	220	0
4000	330	100	25	0

NOTE: all measurements are in microns, obtained by optical micro-meter measurements. Accuracy is ± 6 microns.

Figure 12. Structure of notochord sheath of 4 cm. embryo. Inner layer (I), Middle Layer (M), Outer layer of matrix and mesenchyme (O). ABPAS, x 465.

Figure 12a. Notochord structure in mid trunk region of 4 cm. embryo. ABPAS, x 192.

Figure 13. Structure of notochord of 4 cm. embryo 150 microns from anterior end of notochord. Azure A pH 4.0, x 690.

Figure 14. Structure of notochord sheath 350 microns from anterior end of notochord. Azure A pH 4.0, x 580.

Figure 15. Structure of notochord sheath 1500 microns from anterior end of notochord. Azure A pH 4.0, x 450. Note proximity to floor of hind-brain (HB).

Figure 16. Structure of notochord sheath 2200 microns from anterior end of notochord. Peripheral mesenchyme (M) at maximum extent. Notochord adjacent to floor of hindbrain (HB). Azure A pH 4.0, x 314.

Figure 16a. Notochord at 2200 microns from anterior end showing metachromacy of peripheral mesenchyme (M) and extreme orthochromacy of middle layer of sheath (O). Azure A pH 4.0, x 155.

TABLE Va

4 cm. EMBRYO COMPOSITE RESULTS OF HEAD, TRUNK, AND TAIL
(No. of embryos 3)

TREATMENT	Sheath inner surface	Sheath middle layer	Sheath matrix	Sheath mesench.	Epith. ectoderm	Trunk mesoderm, heart mesoderm
Azure A pH 4.0	O+++	O+++++	M+++	O+-M+++	O+++	O+ M+
Azure A pH 1.5	O±	M++	M+++	- M±	-	O± O±
Hyalase A.A pH 4.0	O±	O+-M±	-	-	O±	- -
Hyalase A.A pH 1.5	-	O±-M±	-	-	O±	- -
Methylate A.A pH 4.0	-	-	-	-	-	- -
Methylate Saponify A.A pH 4.0	-	O±-O+	-	-	-	- -
Diastase A.A pH 4.0	O+	M++	M±-M+	O±-M+	O+	O± O±
0.6 M NaCl A.A pH 1.5	-	-	-	-	-	- -
ABPAS	PAS+++	ABP++++	AB+++	AB+++	ABP++++	AB++ PAS++++
Diastase ABPAS	PAS+	AB+++	AB+	AB+-++	AB++	AB++ -
Saunders 1	+	++++	++++	++	-	++ NP
Saunders 2	±	+++	++	±	-	± NP
Saunders 3	-	±	-	-	-	- NP

Adjacent to the inner surface is a uniform layer about 5 microns thick which shows extreme azurophilia at pH 4 (orthochromatic), metachromasia at pH 1.5, and slight metachromasia at pH 4 and 1.5 following hyaluronidase digestion. Diastase reduces the extreme azurophilia to a moderate metachromacy similar to that at pH 1.5. These results indicate the presence of hyaluronic acid, C-S-A/C, and C-S-B. The restoration of only very slight azurophilia at pH 4 following the methylation-saponification sequence indicates that most staining is due to sulphate groups. Extraction with 0.6 M NaCl abolished all staining indicating that no heparin was present but the presence of a slight amount of heparin was suggested by Acridine orange staining (Saunders 3). It is possible that over-sulphated C-S-C may be binding with Acridine orange as C-S-C containing an extra sulphate group is known to occur in shark cartilage (Dorfman, 1963). The presence of considerably hyaluronic acid and chondroitin sulphates is supported by Acridine orange results.

The extreme orthochromatic reaction of this middle layer with Azure A at pH 4 reflects dye-binding so dense that the metachromatic shift is overcome and orthochromacy appears. This effect may be induced by oversulphation (Pearse, 1961) and has occurred in sulphation of yolk in disc stage embryos (Table I).

The third layer of the sheath consists of mesenchyme cells around the sheath and an area of AMPS appearing as a

matrix associated with the mesenchyme. On the lateral surface of the sheath the mesenchyme was in 2-3 layers with considerable matrix but on dorsal and ventral surfaces there was only 1 layer of mesenchyme and little matrix. Results indicate that the matrix is composed mostly of C-S-A/C with hyaluronic acid as a lesser component. No neurtral polysaccharides are present and it appears that the sheath matrix is a more concentrated form of the pre-cartilage AMPS in the lateral mesenchyme of the anterior notochord.

Epidermal cells may contain acidic polysaccharides according to Azure A and ABPAS results. Acridine orange fails to indicate the presence of any AMPS.

Both trunk and heart mesoderm may contain minor quantities of hyaluronic acid and C-S-A/C but results are not definite. Heart mesoderm does have a quantity of glycogen which is demonstrated by a lability to short incubation with diastase. Basement membrane structures are present in heart and gut areas but they do not show AMPS characteristics.

A dense concentration of PAS positive material was noted in parts of the epithelial ectoderm and in the lumina of branchial filaments. This proved to be glycogen by a lability to short (25 min.) diastase digestion (Figures 17 and 18). This was not noted in any developmental stages other than the 4 cm. stage.

Figure 17. Glycogen deposits (G) in branchial filaments of 4 cm. embryo. ABPAS, x 160.

Figure 18. Branchial filaments of 4 cm. embryo following short (25 minutes) diastase digestion. ABPAS, x 160.

7 cm. Embryo

In the 7 cm. embryo the notochordal sheath has expanded to 6-8 layers of mesenchyme with a like increase in matrix. In trunk and tail, neural and haemal arches are partially established and considerable muscle tissue is differentiating in the tail.

Results indicate the presence of C-S-A/C and C-S-B in the notochord sheath, C-S-A/C in the sheath matrix and in skeletal cartilages. Little hyaluronic acid is indicated in these areas. In contrast, some strongly acidic AMPS may be present in gut, heart and mesonephric tubule mesoderm but results indicate primarily hyaluronic acid (Table VI). Any AMPS present in mesodermal structures in this stage occurs not as a basement membrane, but as fine fibrous networks between cells.

There is indication of some AMPS-like material in neural ectoderm (neural tube), in the ventro lateral part of the mantle (area surrounding the ependymal layer) particularly in cells resembling neurons (Figure 19). Results indicate hyaluronic acid and lesser amounts of C-S-A/C, both of which have been reported present in neurons of bovine brain and spinal cord by Szabo and Roboz-Einstein (1962).

A comparison of results from head and tail regions (Table VII) shows differences in composition of sheath and cartilage matrix between the two regions. C-S-B is found

TABLE VI

7 cm. EMBRYO AZURE A pH AND ENZYME TREATMENTS TRUNK REGION
(No. of embryos 5)

TREATMENT	Sheath matrix	Cart. matrix	Trunk* mesoderm struct.	Trunk mesench.	Neural ectod.
Azure A pH 4.0	M++++	M++++	M++	M+	O+++-M ₊
Azure A pH 1.5	M++	M++	O+	-	O ₊
Hyalase A.A pH 4.0	M+	***	***	***	***
Hyalase A.A pH 1.5	O ₊	-	-	-	-
Methylate A.A pH 4.0	-	-	-	-	-
Methylate Saponify A.A pH 4.0	-	-	-	-	-

* Mesoderm of gut tube, (intestine), heart, epimyocardium, and mesonephric tube and tubules.

** Cytoplasm is unstained in these regions but all nuclei are uniform O++.

Figure 19. Portion of neural tube of 7 cm. embryo, showing Hyaluronic acid (HA) in mantle layer. Azure A pH 4.0, x 128.

TABLE VII

7 cm. EMBRYO COMPARISON OF HEAD AND TAIL CARTILAGE REGIONS
(No. of embryos 3)

TREATMENT	TAIL			HEAD		
	Inner sheath	Sheath matrix	Cart. matrix	Inner sheath	Sheath matrix	Cart. matrix
Azure A pH 4.0	M++++	M++++	M+++	M+	M++++++	M++
Azure A pH 1.5	M++	M++	M++	<u>M±</u>	M+---	M+---
Hyalase Azure A pH 4.0	<u>M±</u>	<u>M±</u>	-	-	M++	- <u>M+</u>
Hyalase Azure A pH 1.5	<u>M±</u>	<u>M±</u>	-	-	M++	- <u>M+</u>
Diastase Azure A pH 4.0	M+	M+++	M+++	-	M++++	M++
Methylate Azure A pH 4.0	-	-	-	-	-	-
Methylate Saponify Azure A pH 4.0	-	-	-	-	-	-

in the inner sheath in the tail but not in the head. A larger proportion of C-S-B is indicated in the sheath matrix in the head region than in the tail. Skeletal cartilage matrix stains more intensely in the tail region than in the head and some C-S-B is indicated in this cartilage in head regions but not in the tail. Skeletal cartilage in the tail consists of developing neural and haemal arches which are associated with the notochordal sheath but in the head, skeletal cartilages include elements of branchial arches, chondrocranium and pectoral girdle which are not necessarily associated with the notochord. The indicated differences in composition may indicate some structural differences in the cartilages or, they may indicate an antero-posterior gradient in complexity of cartilage.

14 cm. Embryo

The area examined in the 14 cm. stage was the notochord and surrounding vertebral elements. Sections included epithelial ectoderm (epidermis), neural tube, notochord and the underlying intestinal epithelium which was closely applied to the bottom of the notochord in the posterior trunk region (Figures 20, 21, 22).

Two of the specimens examined had just hatched and were free swimming. However these could still be

Figure 20. Portion of vertebral column of 14 cm. embryo, showing skeletal elements. (epidermis ep, interdorsal i, basidorsal b, rib r, basiventral v, spinal cord s) ABPAS, x 34.

Figure 21. Longitudinal section through epidermis and underlying vertebral column of 14 cm. embryo. (unicellular mucous gland u, pigment cells p, muscle and connective tissue m, cartilage matrix C) Azure A pH 4.0, x 235.

Figure 22. Cross section through intestinal epithelium and basiventral cartilage of 14 cm. embryo, (blood vessel b) Azure A pH 4.0, x 235.

considered embryos because of the presence of yolk in the intestine.

In the skeletal cartilage around the notochord, interdorsal, basidorsal, interventral, basiventral and rib elements are present. The notochord is completely surrounded by cartilage and centra are developing segmentally. Development of the vertebral column is not complete as the neural tube is still twice the diameter of the centra.

In the cartilage it is clear that chondroitin sulphates A/C are responsible for most staining. C-S-B is indicated in high concentration in epidermal glandular structures and possibly in very slight amounts in cartilage matrix and intestinal glandular structures (Table VIII). The results of Acridine orange staining for chondroitin sulphates (Saunders 2) supports the presence of chondroitin sulphates in all these areas but Azure A staining at pH 4 following extraction with 0.3 M NaCl supports the presence of chondroitin sulphates in cartilage matrix and epidermal glandular structures only. Both Acridine orange staining (Saunders 3) and Azure A staining at pH 4 following extraction with 0.6 M NaCl indicate the presence of variable amounts of heparin, the former in cartilage matrix and epidermal mucins and the latter in cartilage matrix only.

The reduced staining with Azure A at pH 4 following diastase incubation and the almost complete loss of azurophilia at pH 1.5 following hyaluronidase incubation

TABLE VIII

14 cm. EMBRYO SKELETAL CARTILAGE AND EPITHELIA
(No. of embryos 3)

TREATMENT	Cart. matrix	Epid. mucins (glands)	Intest. mucous glands
Azure A pH 4.0	M++++++	M++++	M++++
Azure A pH 1.5	M++	M++++	M++
Hyalase A.A pH 4.0	- <u>M</u> ±	O++++++	O++++
Hyalase A.A 1.5	- <u>O</u> ±	O+-++++	- <u>O</u> ±
Diastase A.A pH 4.0	M++++	M++++	O++++++
Methylate A.A pH 4.0	-	-	-
Methylate Saponify A.A pH 4.0	-	-	-
ABPAS	AB+-++++	AB++++*	AB++++*
Diastase ABPAS	AB++++**	AB++++**	AB++++**
Saunders 1	++	+++	+
Saunders 2	++	+++	+
Saunders 3	±-+	±-+	±
0.3M NaCl A.A pH 4.0	O+-M++	- <u>O</u> ++	-
0.6M NaCl A.A pH 4.0	- <u>M</u> ±	-	-

* Staining is uneven, extremely variable.

** Staining is uniform.

do not support the presence of C-S-B in intestinal gland structures. These results infer that some material other than AMPS is present.

The material in epidermal gland structures which stains intensely with Azure A at pH 1.5 following hyaluronidase digestion can only be C-S-B. Seno and Meyer (1963) reported that C-S-B was the major sulphated polysaccharide of shark skin.

Staining results indicate that C-S-A/C are the major components of cartilage matrix and, that C-S-B is present primarily in epidermal mucous glands and in variable amounts in cartilage matrix. Variable Alcian blue staining of cartilage matrix which became uniform and more intense if it was preceded by diastase digestion (Figures 23, 24) indicated that some polysaccharide materials are present in matrix which decrease the availability of binding sites in different areas. The indicated presence of C-S-B in matrix may be due to such an interference in staining mechanism.

Figures 25 to 30 are included to show some of the results of cartilage matrix staining which are presented in Table VIII.

Figure 23. Cartilage matrix from 14 cm. embryo, untreated, ABPAS, x 450.

Figure 24. Cartilage matrix from 14 cm. embryo, long (2 hours) diastase digestion, ABPAS, x 450.

Figure 25. Cartilage of 14 cm. embryo. Azure A pH 1.5, x 450.

Figure 26. Cartilage of 14 cm. embryo. Extracted with 0.3 M NaCl, Azure A pH 4.0, x 450.

Figure 27. Cartilage of 14 cm. embryo, extracted with 0.6 M NaCl, Azure A pH 4.0, x 600.

Figure 28. Section of epidermis and cartilage of 14 cm. embryo, hyaluronidase digestion, Azure A pH 4.0, x 130.

Figure 29. Section of epidermis and underlying tissues of 14 cm. embryo, (unicellular mucous gland U, pigment cells P, cartilage matrix M), Azure A pH 4.0, x 238.

Figure 30. Epidermis and underlying tissues of 14 cm. embryo, extracted with 0.6 M NaCl, Azure A pH 4.0, x 130.

DISCUSSION

Specificity of Staining Methods

Specificity of staining techniques is a problem pertinent to any histochemical study. In this instance three stains were used to demonstrate AMPS; the Alcian blue component of the ABPAS procedure, Azure A, and Saunders' fluorescent Acridine orange method. Satisfactory agreement between all three stains was achieved in cleavage stage embryos through 2-4 mm. and 6-7 mm. stages. Material that was PAS positive did not react with the other stains and material demonstrated as hyaluronic acid by Acridine orange fluorescence was Alcian blue positive and metachromatic with Azure A. Through the first three developmental stages studied there was no difficulty in interpreting the results of the staining treatments.

However, in the 17-18 mm. stage there appeared, with the first indication of sulphated AMPS, some variation in Alcian blue responses. Methylation is expected to block both sulphate and carboxyl groups by desulphating the former and forming methyl esters on the latter. Demethylation removes the methyl esters and restores carboxyl staining only. Methylation did not block Alcian blue staining but subsequent demethylation abolished alcianophilia entirely.

Acetylation and deacetylation is designed to block hydroxyl and amino groups and then to restore hydroxyl groups only. This procedure is applicable in

identification of neutral and acid polysaccharides. However, it was found that the latter part of this treatment abolished all staining of sulphated AMPS with Alcian blue or Azure A while the acetylation part of the treatment had little or no affect on sulphated AMPS staining. A possible explanation is that hydroxyl groups are substituted in place of acidic groups and thus will not stain with Alcian blue or Azure A when sulphated AMPS are subjected to this treatment. Yamada (1964) observed similar results using known sulphated AMPS and Alcian blue-PAS staining. A new staining method for Alcian blue developed by Scott, Quintarelli and Dellovo (1964) apparently has overcome such difficulties and allows more specific staining with Alcian blue.

In general it was found that the methylation-demethylation sequence was useful in assessing the degree of staining due to carboxyl and sulphate groups respectively, when Azure A was the stain used in conjunction with this treatment. ABPAS results were not consistent. Acetylation and deacetylation was useful only when used to locate glycogen and neutral polysaccharide staining with ABPAS staining. Alcian blue staining however, was frequently affected by the deacetylation treatment. It may be, as suggested by Yamada (1964) that strongly acidic groups are affected by the alkali used in the deacetylation treatment.

In the 14 cm. embryo, there was conflict between

Acridine orange and Azure A results. The former indicated heparin in epidermal unicellular mucous glands, in cartilage matrix and in intestinal epithelial glands while the latter method indicated heparin in cartilage matrix only. The only basic difference between the two methods is that the Azure A solution is acidified with HCl and Acridine orange with acetic acid. The explanation for the staining difference is probably that chondroitin sulphates and/or hyaluronic acid were still present and staining. This would suggest that protein or some other material interfered with the NaCl extraction. This type of interference was noted in Alcian blue staining of cartilage matrix which was very uneven in control sections and became dense and uniform following diastase treatment. Szirmai (1963) noted protein interference with Azure A staining of horse nasal septum cartilage.

The use of hyaluronidase has been criticized as non-specific (Szirmai, 1963). Walker (1961) and Curran (1961) stated that analyses of the action of commercial bull testis hyaluronidase showed that the enzyme was specific for hyaluronic acid, C-S-A and C-S-C, and several studies of AMPS have used commercial preparations, 'Hyalase', with apparent success in the extraction of those materials (Kato and Sirlin, 1963; Zugibe, 1962; Mancini, Vilar, Stein and Fiorini, 1960). In this study the specificity of hyaluronidase was satisfactory. However, the characterization of a particular AMPS was based on several criteria

in addition to hyaluronidase lability. Relative acidity, resistance to salt extraction at different molarities and blocking and unblocking of specific acid groups were necessary factors in the identification of a specific AMPS.

It became apparent that there are limits to the degree of identification possible. With the techniques employed identification of glycogen, neutral polysaccharides (probably oligosaccharides), hyaluronic acid, chondroitin sulphate A/C and chondroitin sulphate B was achieved. It must be noted that such identifications were the least difficult in the youngest developmental stages. As development proceeds, AMPS containing areas become more complex with the addition of other materials and positive identification of AMPS becomes difficult.

Embryonic Development of AMPS

An examination of the results in the different embryonic stages clearly shows a progression in the appearance of AMPS (Table IX).

Neutral polysaccharides and possibly some acid polysaccharides were the main constituent of cleaving cells. Some hyaluronic acid was suggested but there was not sufficient quantity to permit positive identification. The definite localization of the polysaccharides can be

TABLE IX

GENERAL SUMMARY OF RESULTS FROM TABLES I-VIII LOCATION OF IDENTIFIABLE AMPS

STAGE	Neutral Poly- saccharides	Hyaluronic acid	C-S-A/C-S-C	C-S-B	Heparin
Cleavage	cleavage fur- rows, general cytoplasm.	-	-	-	-
2-4 mm.	ectoderm cells, notochord cells, endoderm cells, mesoderm cells.	ECM, perinotoch. sheath.	-	-	-
6-7 mm.	endoderm cells, mesoderm cells, neural ecto.cells.	ECM, mesoderm cells, neural ecto.cells.	perinotoch. sheath.	-	-
17-18 mm.	notoch.cells, notoch. sheath.	notoch. sheath, mesod. basement membranes, mesenchyme, trunk mesod.	notoch.sheath.	-	-
4 cm.	inner sheath, heart mesod., branchial fil- aments, epidermis.	precart. of para- chordals, peripheral shth. mesenchyme, trunk mesod., heart mesod., mesoneph.tube mesoderm.	precart. of parachordals, middle sheath, periph.sheath mesenchyme.	middle sheath.	-
7 cm.	-	gut, heart mesod., mesoneph.tube mesod, neural tube.	sheath, mid., periph.matrix, skeletal cart., neural tube.	sheath mid.	-
14 cm.	-	cart.matrix.	cart.matrix.	epidermis	-

attributed to a fixation artefact comparable to that of glycogen sometimes seen in liver cells. Cleaving cells are large (0.31 mm. X 0.13 mm.) and yolky and the position of polysaccharides infers that the displacement occurs during precipitation with C-P-B-formalin.

Hyaluronic acid was identified first in the 2-4 mm. embryo. It appeared extracellularly associated with very thin extensions of mesoderm cells to the overlying neural and epidermal ectoderm and as a uniform, thin layer around the notochord. Hyaluronic acid was positively identified in this material but neutral polysaccharides were the main component. Because this extracellular polysaccharide and AMPS material possessed some of the characteristics of the 'Extracellular Material' described by Moscona (1960) and Steinberg (1963), it was labelled ECM. The structure of ECM is a basement membrane like area with extracellular extensions to adjacent tissue layers. The extensions are very similar to the 'pseudopod' structure of migrating mesenchyme cells described by Gustafson and Wolpert (1963) in sea urchin development. Steinberg proposed that ECM is artificially produced from the chromosomes of ruptured cells in dissociated tissues by the action of enzymes used for dissociation. This may be true in systems of dissociated cells. However, there are several reasons which argue against Steinberg's proposal with respect to the appearance of ECM in the whole skate embryo. First, ECM appears only in specific

developmental stages. Secondly, ECM appears in specific embryonic locations and cannot be ascribed to a fixation artefact. In the 6-7 mm. embryo ECM appears between mesoderm and endoderm, between mesoderm and epidermis but does not appear for example between somatic and splanchnic mesoderms. The appearance of ECM in the 2-4 mm. embryo and the 6-7 mm. stage and its specific location suggest a developmental function. There are differences in the form of ECM at different developmental stages which may suggest different functions at different times but which may be artefacts due to histochemical procedures. Most of the ECM in the 2-4 mm. stage appears in association with cell extensions or in 'pseudopod' form between tissue layers but some does appear as the smooth layer form around the notochord. In the 6-7 mm. embryo most ECM is in the smooth layer form but in some areas where tissues have been separated in histological procedures the 'pseudopod' form appears. This suggests that the actual ECM appearance would be the smooth layer form. Possible functions of this form could be to function in tissue adhesion or to keep tissues separated in a chemical sense. The highly hydrated chemical structure of AMPS in ECM could easily act as a diffusion barrier to large molecules.

Hyaluronic acid is found in the 17-18 mm. embryo in basement membrane like structures around the developing gut tube and mesonephros. This was not included as ECM

because it appeared to be directly concerned with specific organ development but it may function in a manner similar to ECM. Such basement membrane like structures were not found in any later developmental stages. Hyaluronic acid is found throughout development, associated with epimyocardium, Wolffian duct and mesonephric tubules in the 4 cm., and 7 cm. stages but it appears as finely branching processes between mesenchyme or fibroblast cells. In later development, hyaluronic acid persists as a component of cartilage matrix.

The first sulphated AMPS to appear were C-S-A/C around the notochord sheath of the 6-7 mm. embryo. Chondroitin sulphates were not demonstrated in other embryonic areas until the 4 cm. stage. In that stage, C-S-A/C were identified in the matrix of the precartilaginous material of the parachordal chondrocranium elements, in the middle cartilaginous layer of the notochord sheath, and extracellularly in mesenchyme or fibroblasts peripheral to the notochord. In the 4 cm. embryo, C-S-B was identified in the middle layer of the notochord sheath and in the 14 cm. embryo in unicellular mucous glands of the epidermis.

The production of cartilage in the 4 cm. embryo was preceded by a condensation of mesenchyme cells around the notochord sheath, or as a condensation of mesenchyme (fibroblast) cells in a place where skeletal cartilage is to be formed. AMPS then appeared between the closely packed fibroblast cells in a uniform matrix. No neutral

polysaccharides were demonstrated in association with these cells either before or after the formation of AMPS. AMPS were not identified within the fibroblasts but only in the interstitial matrix. The density of AMPS staining in the matrix seemed to depend on the proximity of fibroblasts one to another. There was no demonstrable morphological difference between fibroblasts surrounding AMPS matrix and those which were not. This agreed with the study of Mancini, Vilar, Stein and Fiorini (1961) which showed in cultures of fibroblasts that sulphate was incorporated into both fibroblasts and intercellular matrix but histochemical tests for AMPS were positive only in intercellular matrix. This may indicate that low molecular weight sulphate containing AMPS precursors are present within fibroblast cells and are polymerized outside the cell to form AMPS.

Some AMPS appeared in locations suggestive of a physiological or biochemical function. Hyaluronic acid and C-S-A/C were identified in one area of the 7 cm. embryo spinal cord, and were not found in spinal cord of more advanced stages. Hyaluronic acid has been biochemically identified in bovine white matter (spinal cord) by Szabo and Roboz-Einstein (1962). They suggested that hyaluronic acid in nerve tissue might function in myelin construction.

The morphological structure of the anterior portion of the notochord and associated parachordal

precursors is of interest from the point of view of adjacent tissue interactions. Table Va shows that an inverse relation exists between the maximum extent of the notochord and the vertical distance from the notochord to the floor of the hind brain. The maximum size of the notochord is reached only when the floor of the hind brain appears to touch the notochord. The extent of the precartilaginous materials of the parachordals may also be related to the distance between notochord and hind brain on one hand and to the maximum dimensions of the notochord on the other. This idea is supported by the study of Glick, Lash and Madden (1964) which showed that chick embryo somites (St. 16-17) cultured in the presence of notochord or spinal cord would contain the enzymes necessary for chondrogenesis but somites cultured alone would not contain these enzymes.

The comparison of cartilage from head and tail regions of the 7 cm. embryo revealed slight differences in composition. Cranial region cartilage stained with greater intensity and showed more advanced characteristics e.g. greater content of C-S-B, lack of neutral polysaccharides, than the caudal cartilage. However, there were no other examples of such an antero-posterior gradient noted.

The proposed identification of heparin in cartilage matrix and epidermal unicellular glands in the 14 cm. embryo by both Azure A and Acridine orange results can not be considered positive. The extractive

techniques used in this procedure are suspect and it is more likely that sulphated AMPS bound by protein in cartilage matrix were not extracted and thus stained as heparin. Mathews (1962) in a biochemical study of shark cartilages and bovine cartilages noted a 12% protein content in adult shark cartilage. Another reason to suspect the indicated presence of heparin is that it has not been identified in cartilage to date.

In review, there appears in the development of Raja binoculata a progression from neutral polysaccharides in cleavage stages to weakly acidic AMPS in neural tube stages and finally to strongly acidic sulphated AMPS in more advanced stages of cartilage development.

Neutral polysaccharides are intracellular and persist in certain areas until the 4 cm. stage. Their presence in cleaving cells relates to the high yolk content of these first formed cells. In the 2-4 mm. and 6-7 mm. stages the gradient of neutral polysaccharides relates to morphogenetic activities of different tissues occurring at different rates and might possibly indicate the presence of oligosaccharide precursors for AMPS production (Figure 30).

Hyaluronic acid, the weakly acidic AMPS occurring with neural polysaccharides in stages of neurulation and extension of major organ forming areas in the form of ECM, may function as an agent of tissue adhesion, of tissue movement, or as a medium governing some chemical

aspect of the extracellular space. In stages of organ system development hyaluronic acid appears to be associated with specific organ systems in a form similar to ECM but without the neutral polysaccharide component. Hyaluronic acid persists through development and adult stages as a component of cartilage matrix.

Chondroitin sulphates A and C participate primarily in cartilage formation and persist in adult stages as the major AMPS component of cartilage matrix. Their function is structural and they may participate in some aspects of neural tissue development. Chondroitin sulphate B appears as a structural layer of the notochord and in unicellular mucous glands of the epidermis in the advanced embryo. It may also be a component of cartilage matrix in the very advanced embryo.

There is no obvious explanation for the differences in function and localization of the various AMPS discussed.

The results obtained in this study agree with previous biochemical analyses of shark skins and cartilages cited elsewhere.

TABLE X

METABOLIC STEPS IN THE BIOSYNTHESIS OF CHONDROITIN SULPHATES

from Kent, P. W. Some Biochemical aspects of Sulphated Mucopolysaccharides,
Biochemical Society Symp. No. 20, Cambr. Univ. Press, 1961.

SUMMARY

1. Embryonic stages of the elasmobranch, Raja bionculata, ranging from cleavage to immediate prehatching were examined by histochemical techniques to follow acid mucopolysaccharide (AMPS) development.
2. A progression was observed from (1) intracellular neutral polysaccharides in cleaving stage to, (2) a combination of extracellular neutral polysaccharides and weakly acidic acid mucopolysaccharides (hyaluronic acid) associated with cell processes in neurulating embryos to, (3) extracellular strongly acidic sulphated acid mucopolysaccharides (chondroitin sulphates) in later stages, particularly in areas of cartilage development.
3. Hyaluronic acid first appeared in the early neurula in an extracellular form associated with cell processes and, as a smooth layer around the notochord and on the ventral surface of neural and epidermal ectoderm. Neutral polysaccharides were associated with this extracellular material (ECM) which occurred between some adjacent tissue layers but not between somatic and aplanchnic mesoderm. In post neurulae, hyaluronic acid appeared in a basement membrane like form associated with the development of mesonephric and gut tubes and as a thin layer around the notochord. In older embryos hyaluronic acid appeared in

mesenchyme associated with heart and mesonephric tubule development, in fibroblast produced precartilage matrix and in cartilage matrix.

4. Sulphated AMPS appeared only in later stages (17-18 mm. to immediate prehatching) particularly in areas of cartilage development. C-S-A/C were identified in the notochord sheath, in associated sheath mesenchyme (fibroblasts) and precartilage matrix and in skeletal cartilage matrix. C-S-B appeared in the middle layer of the notochord sheath and in the unicellular epidermal mucous glands.
5. Both hyaluronic acid and C-S-A/C were identified in neuron like cells in the mantle area of the neural tube of the 7 cm. embryo.
6. Identification of AMPS using the histochemical techniques described is least difficult in early embryonic stages. In later developmental stages, it becomes difficult to identify AMPS specifically, particularly in cartilage matrix.
7. The results obtained in immediate prehatching stages agreed with previously reported biochemical analyses of adult shark skins and cartilages.

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APPENDIX A

Structure of Acid Mucopolysaccharides

Hyaluronic acid

Polymer of glucuronic acid and N-acetylglucosamine, beta 1:3 glycosidic linkage, testicular hyaluronidase labile.

Chondroitin sulphate A (C-S-A)

Polymer of glucuronic acid and N-acetylgalactosamine, beta 1:3 glycosidic linkage, one sulphate group at C 4 position, testicular hyaluronidase labile.

Chondroitin sulphate B (C-S-B)

Polymer of iduronic acid and N-acetylgalactosamine, beta 1:3 glycosidic linkage, one sulphate group at C 4

position, not labile to testicular hyaluronidase.

Chondroitin sulphate C (C-S-C)

Polymer of glucuronic acid and N-acetylgalactosamine, beta 1:3 glycosidic linkage, one sulphate group at C6 position, testicular hyaluronidase labile.

Heparin

Polymer of glucosamine and glucuronic acid, two sulphate groups at C 2 and C 4 positions, may have an additional sulphate, glycosidic linkage alpha 1:3 or possibly alpha 1:4, not labile to testicular hyaluronidase.

APPENDIX B

Histochemical Methods Employed

A. Fixation (Williams and Jackson, 1956)

Fix small pieces of tissue in 0.5% cetylpyridinium-chloride (Bromide proved satisfactory) in 4% aqueous formaldehyde at room temperature. Wash well in running water before dehydration. (Alternate nomenclature is hexadecyltrimethyl-ammonium chloride or bromide, CPC or CPB.)

B. Staining

1. Azure A (Szirmai, 1963)

Stain for 30 minutes in 0.2% Azure A (Fisher Scientific) in distilled water with pH adjusted to 4.0 or 1.5 with HCl. Rinse in distilled water, dehydrate in air, clear, mount in permount.

2. Alcian blue PAS (Spicer, 1960)

Stain for 30 minutes in 1.0% Alcian blue (G. T. Gurr) in 3% acetic acid, rinse in running water for 10 minutes, follow with Periodic acid Schiff treatment, water rinse, alcohol dehydration, mount in permount.

3. Acridine orange (Saunders, 1964)

Treat 3 parallel slides in 1.0% CPC or CPB for 5 minutes, wash in running water for 10 minutes, treat with ribonuclease 1 mg/ml in glass

distilled water at 45°C for two hours.

Treat slide 1 in CPB again, wash in running water for 10 minutes, stain for 3 minutes in 0.1% Acridine orange (G. T. Gurr) in distilled water.

Stain slide 2 for 5 minutes in 0.1% Acridine orange in 0.01 M acetic acid (pH 3.2), wash in running water for 10 minutes, differentiate in 0.3 M NaCl in 0.01 M acetic acid for 10 minutes.

Treat slide 3 as slide 2, but differentiate in 0.6 M NaCl in 0.01 M acetic acid for 10 minutes.

It may be necessary to prolong differentiation up to 30 minutes, until stain does not come out of the sections. All slides are washed well in running water, air dried and mounted in Flourmount (E. Gurr).

To interpret, in slide 1 red fluorescence is due to hyaluronic acid, in slide 2, to chondroitin sulphates and heparin and in slide 3, to heparin.

C. Enzyme treatments

1. Hyaluronidase (Pearse, 1961; Culling, 1963)

Incubate sections for 3 hours at 37°C in 1 mg/ml hyaluronidase in 0.85% saline, wash well in distilled water. Control sections are

incubated in 0.85% saline.

AMPS staining in control slides but not in enzyme treated slides is hyaluronic acid, C-S-A/C or a mixture of these compounds. (NBC testicular hyaluronidase specified.)

2. Diastase (Culling, 1963)

Digest sections in 0.1% commercial malt diastase in glass distilled water for 30 minutes at 37°C to remove glycogen. Oligosaccharides and all carbohydrates may be removed if incubation is prolonged from 3 to 24 hours. Control slides are incubated in distilled water but this may remove a large portion of the glycogen present.

D. Salt extractions (Kelly, Bloom and Scott, 1963; Saunders, 1964)

1. Treat sections for 10 minutes (longer if necessary), in 0.3 M NaCl in 0.01 M acetic acid at room temperature, rinse well in running water, stain as required. AMPS staining will consist of chondroitin sulphates and/or heparin.
2. Treat sections as before but in 0.6 M NaCl in 0.01 M acetic acid. AMPS staining will be heparin.

For this treatment (1 and 2) CPB/C-formalin is a required fixative.

E. Selective blocking and unblocking treatment

1. Methylation-demethylation (Fisher and Lillie, 1954; Spicer, 1960; Yamada, 1964)

Treat ethanol dehydrated sections for 1-4 hours at 60°C in preheated methanol made 0.1 N with HCl. Rinse well in ethanol, hydrate and stain.

Methylate parallel sections, celloidize, and treat in 1% KOH in 70% ethanol at 25°C for 30 minutes, rinse well in ethanol, hydrate and stain.

Methylation hydrolyzes sulphage groups and forms methyl esters on carboxyl groups thus blocking all acid groups on AMPS. Demethylation removes the methyl esters from the carboxyl groups thus restoring staining to carboxyl groups only.

2. Acetylation-deacetylation (Pearse, 1961)

Treat uncoated dehydrated sections in 40% acetic anhydride in anhydrous pyridine for 1-24 hours at 25°C or for one half to six hours at 60°C.

To saponify or deacetylate, rinse acetylated sections well in ethanol, celloidize, and treat in 0.1 N KOH in 70% ethanol for 45 minutes at room temperature.

Acetylation blocks hydroxyl groups with acetyl esters and may hydrolyze alpha amino groups thus blocking PAS staining. Deacetylation removes

the esters and restores hydroxyl group PAS staining only.

3. Sulphation (Kramer and Windrum, 1954; Spicer, 1960)

Treat uncoated dehydrated sections in a coplin jar (screw top) containing 10 ml. of concentrated sulphuric acid and 30 ml. of glacial acetic acid for 10-20 minutes at room temperature. Rinse well in ethanol, hydrate and stain.

Sulphation induces sulphate esters on hydroxyl groups carried on polysaccharides.