OBSERVATIONS ON THE ACID GLYCOSAMINOGLYCAN (MUCOPOLYSACCHARIDE)* CONTENT OF THE MATRIX OF AGEING CARTILAGE

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The changes which occur with age in joint cartilage (Davies, 1961) and ordinary hyaline cartilage (Silberberg and Silberberg, 1961) have been reviewed recently. Chemical studies of the glycosaminoglycan content of the matrix have shown that while the total carbohydrate content of the cartilage diminishes with aging, keratosulphate shows a linear increase (Kaplan and Meyer, 1959). The method of Scott, Dorling, and Quintarelli (1964), employing a new Alcian blue technique, offers an approach to the problem of the localization of keratosulphate within the matrix. As changes in the matrix of articular cartilage are acknowledged to be among the first to be observed in the ageing of the tissue (Bennett, Waive, and Bauer, 1942), information about keratosulphate should be of interest in the elucidation of the aetiology of joint disease. To this end articular as well as ordinary hyaline cartilage have been examined and compared.

Material and Methods

Material

Human *post-mortem* material was used. Specimens of the 4th costal cartilage and of articular cartilage from the medial femoral condyle were taken; areas free of macroscopic erosions were sampled. The ages of the subjects were 1½, 13½, 18, 31, 34, 60, 64, 69, and 78 years. In addition, a specimen of articular cartilage from the head of the humerus of an amputated limb from a person aged 50 years was also examined.

Methods

Blocks were fixed for 3 days at -20° C. in alcoholic formalin (1 part 40 per cent. formaldehyde: 9 parts

ethanol), carried through two changes, each for 24 hrs, of absolute alcohol, and of xylol (at -20° C.). The final change in xylol was allowed to warm to room temperature, before routine embedding in paraffin wax, and sectioning at 10μ. Sections were dewaxed with xylol and allowed to dry on the slide at room temperature before staining. Sections were stained as set out below:

- (1a) Alcian blue (AB) 0·1 per cent. in 0·05M acetate buffer pH 5·8 containing magnesium 0·4M chloride (referred to below as AB—0·4M MgCl₂) for 4 hrs at room temperature.
- (1b) As for (a), but with 0·9M instead of 0·4M magnesium chloride (AB—0·9M MgCl₂).
- (2) Alcian blue (AB) 0·1 per cent. brought to pH 0·7 with hydrochloric acid, for 30 min.
- (3) Azure A, 2mM, at pH 2·0 for 15 min.
- (4) Bismarck brown 1·0 per cent. in 1 per cent. acetic acid for 1 min.
- (5) Periodic acid-Schiff reaction, including diastase controls for glycogen.
- (6) Haematoxylin and eosin.

After staining with the cationic dyes, sections were examined direct from the staining solution before rinsing with water, to exclude artefacts due to the rinse causing changes in pH or Mg++ ion concentration while the dye was still available to the tissue.

Results

The matrix has been considered to consist of zones according to its relationship to the cell lacuna:

- (i) Territorial.—A zone of matrix surrounding one cell or group of cells.
- (ii) Interterritorial.—The region between territorial zones.

* The terminology of Jeanloz (1960), preferable to the older nomenclature, is used throughout.

It is also convenient to consider the tissue in the gross terms of subperichondral, peripheral, and central regions for costal cartilage, and superficial and deep regions for articular cartilage.

In general it is found that the staining with AB —0.4M MgCl_2 and at pH 0.7, and with Azure A and Bismark brown is similarly localized, although there are slight differences in intensity. This group of techniques contrasts with the use of AB —0.9M MgCl_2 . The results of the haematoxylin and eosin preparations are in agreement with the classical descriptions of the tissue.

Hyaline Costal Cartilage.—In the central region of ageing costal cartilage, AB —0.9M MgCl_2 and all the cationic dyes (Fig. 1*a, b*) stain the territorial areas intensely against the more lightly stained interterritorial matrix. A narrow band is sometimes stained more intensely in the middle of the latter. This type of staining pattern is seen from the later part of the second decade into old age. In the

peripheral region of ageing costal cartilage, the interterritorial zone is sometimes more difficult to discern, but with most of the cationic dyes the territorial area is stained well (Fig. 2*a*, opposite), leaving the interterritorial area a lighter colour. Using AB —0.9M MgCl_2 , the territorial matrix is unstained (Fig. 2*b*, opposite).

Similarly, the subperichondral matrix is stained by all procedures except AB —0.9M MgCl_2 .

In young tissue the staining pattern is observed to be more variable in the central region of costal cartilage than in the aged tissue. This may be accounted for partly by the greater cellularity of the younger tissue and partly by the fact that the staining pattern of the territorial zone is not always consistent. Thus, the staining may be similar in pattern to the "aged" type or, conversely, the territorial may be less intensely coloured than the interterritorial matrix (Fig. 3*a*, opposite). However, AB —0.9M MgCl_2 usually stains only the interterritorial area (Fig. 3*b*, opposite).

(*a*) Stained with AB—0.4M $\text{MgCl}_2 \times 120$

(*b*) Stained with AB—0.9M $\text{MgCl}_2 \times 120$

Fig. 1.—Central region of costal cartilage. Aged 64 years. The territorial staining is similar at both electrolyte concentrations.

(a) Stained with AB—0.4M $\text{MgCl}_2 \times 120$

(b) Stained with AB—0.9M $\text{MgCl}_2 \times 120$

Fig. 2.—Peripheral region of costal cartilage. Aged 64 years. Only interterritorial staining occurs at the higher electrolyte concentrations.

(a) Stained with AB—0.4M $\text{MgCl}_2 \times 120$

(b) Stained with AB—0.9M $\text{MgCl}_2 \times 120$

Fig. 3.—Central region of young costal cartilage. Aged 13 years. Predominantly interterritorial staining occurs at the higher electrolyte concentrations.

In the peripheral region of young costal cartilage, although the majority of the cationic dyes give a diffuse effect with a slight falling off in intensity in the interterritorial zone (Fig. 4a), only the latter is stained using AB—0.9M MgCl_2 ; a rather striking "network" pattern is produced (Fig. 4b). The subperichondral matrix is not stained by any of the procedures. In infant costal cartilage there is hardly any staining when AB—0.9M MgCl_2 is used, although a dense colour is produced with the other procedures, including Bismarck brown.

It is interesting that, after staining with the cationic dyes (particularly Azure A), at low pH, a narrow unstained band is often seen between the territorial and interterritorial areas. This is observed only infrequently using Alcian blue with magnesium chloride at pH 5.8.

Hyaline Articular Cartilage.—With all techniques the intensity of staining falls to zero in passing from the deep part of the tissue to the articular surface. Most of the cationic dyes stain the territory around the chondrocytes of the superficial (Fig. 5a, opposite) and the deep region, although there is some variability between the specimens in the distance from the articular surface at which this staining occurs. However, aged articular cartilage differs from aged costal cartilage in that staining with AB—0.9M MgCl_2 in all regions of the tissue is predominantly interterritorial, although some slight territorial staining is seen in the deepest parts near the calcified layer (Figs 5b and 6, opposite).

In younger articular cartilage, the superficial matrix is more intensely stained than in the ageing tissue. Further, the territorial is less well stained

Fig. 4.—Peripheral region of young costal cartilage. Aged 13 years. Marked interterritorial staining occurs at the higher electrolyte concentrations

(a) Stained with AB—0.4M MgCl_2 $\times 120$

(b) Stained with AB—0.9M MgCl_2 $\times 120$

Fig. 5.—Superficial region of articular cartilage of head of humerus. Aged 50 years.

Note elimination of territorial staining in (b).

than the interterritorial matrix in the superficial region. AB—0.9M MgCl_2 is again localized solely to the interterritorial matrix (Figs 7a, b, overleaf).

Periodic Acid-Schiff Results.—The use of diastase affects only the staining of the intracellular glycogen. In the matrix it is interesting that the more intense PA-S positivity is seen in the localities which stain with AB—0.9M MgCl_2 , although the subperichondral zone in the younger age group is Schiff-positive also. The PA-S reaction appears to be absent or much reduced in the territorial areas of the superficial region of ageing articular cartilage.

The results are summarized on a 0 $\rightarrow$ +++ basis in Tables I and II (overleaf).

Fig. 6.—Full thickness of articular cartilage of medial femoral condyle. Aged 60 years. Stained with AB—0.9M MgCl_2 $\times 120$
Note generalized interterritorial staining except in deep zone where territorial staining occurs also.

Discussion

The present Alcian blue technique, as introduced by Scott, Dorling, and Quintarelli (1964), depends on magnesium chloride in the staining solution at

(a) Stained with AB—0.4M MgCl_2 $\times 120$ (b) Stained with AB—0.9M MgCl_2 $\times 120$

Fig. 7.—Superficial region of articular cartilage of medial femoral condyle. Aged 18 years. Note absence of territorial staining in both techniques.

critical concentrations to stain selectively. The method is an extension of the analytical techniques used for the fractionation of cetylpyridinium—glycosaminoglycan complexes (Scott, 1955, and 1960) by solution in various concentrations of salt. In tissue sections, substances binding AB at 0.4M MgCl_2 include chondroitin sulphate, heparin, and keratosulphate; at 0.9M MgCl_2 , only high molecular weight keratosulphate binds Alcian blue, among the compounds tested (see *e.g.* Laurent and Scott, 1964, for a discussion of the molecular weight factor in similar precipitation reactions). Heparin does not occur in cartilage and hence this procedure offers a method of distinguishing histochemically between chondroitin sulphate and high molecular weight keratosulphate; in the present study it is used particularly for the localization of high molecular weight keratosulphate. The results indicate that high molecular weight keratosulphate is located as follows:

In the peripheral region of ageing costal cartilage, in both regions of young costal cartilage, and in articular cartilage, keratosulphate lies only in the interterritorial matrix; in aged articular cartilage there may be a little present in the territorial areas of the deepest part of the tissue, next to the calcified layer. In the central region of ageing costal cartilage, it lies in the territorial areas, and to judge by the intensity of staining with Alcian blue at low and high electrolyte concentration (see Table I), it may be the major glycosaminoglycan represented here. The interterritorial matrix possesses some keratosulphate.

These conclusions, like those of the earlier workers in the field (Silberberg and Silberberg, 1961), are at variance, so far as the central region of ageing hyaline (costal) cartilage is concerned, with the microchemical results on horse nasal cartilage obtained by Szirmai (1963), who claims that neither the concentration nor the composition of the glycosaminoglycan differs significantly between the area of the "chondrones" and the interterritorial area.

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TABLE I
STAINING PROPERTIES OF THE MATRIX OF HUMAN COSTAL CARTILAGE

Type	Region	Area	Alcian Blue			Azure A	Bismarck Brown	Periodic Acid-Schiff
			0.4M MgCl ₂	0.9M MgCl ₂	pH 0.7			
Old	Central	Territorial ..	+++	+++	+++	+++	+++	++
		Interterritorial ..	+	+	+	0	±	+
Young	Peripheral	Territorial ..	++	0	++	+++	+++	±
		Interterritorial ..	+	++	++	++	+++	++
Young	Central	Territorial ..	0→++	0	0→++	0→++	+	+
		Interterritorial ..	++	++	+	+++	++	++
Young	Peripheral	Territorial ..	+++	0	++	++	+	+
		Interterritorial ..	++	++	+	++	+	++

TABLE II
STAINING PROPERTIES OF THE MATRIX OF HUMAN ARTICULAR CARTILAGE

Type	Region	Area	Alcian Blue			Azure A	Bismarck Brown	Periodic Acid-Schiff
			0.4M MgCl ₂	0.9M MgCl ₂	pH 0.7			
Old	Superficial	Territorial ..	++	0	++	+++	+++	0
		Interterritorial ..	+	+	+	+	+	+
Old	Deep	Territorial ..	++	0	++	+++	+	+
		Interterritorial ..	++	++	++	+++	+++	++
Young	Superficial	Territorial ..	+	0	+	+	+	0
		Interterritorial ..	++	++	++	++	+	+
Young	Deep	Territorial ..	++	±	++	+++	+	0
		Interterritorial ..	++	++	+	++	+++	++

Possible reasons for the discrepancy are:

- The use by Szirmai of horse as opposed to human cartilage,
- The use of hyaline cartilage from a different part of the body,
- The difficulties of obtaining homogeneous samples by microdissection,
- Variations between areas in the water content content of frozen dried sections.

The first three factors are self-evident, but the fourth may well be equally important. Solutions of acid glycosaminoglycans are very difficult to reduce to complete dryness by freeze drying (Blumberg, Ogston, Lowther, and Rogers, 1958). An area rich in glycosaminoglycan may thus be relatively "wet" in comparison with an area poor in glycosaminoglycan following lyophilization. The two regions are thus compared on different bases, the former having less dry weight/mg. than the latter. In the case of stained sections, there may be similar difficulties in deciding whether the regions are being compared on the same basis, and it would be fortuitous if they were identical.

Attention is drawn to the fact that Alcian blue at 0.9M MgCl₂ is specific for *high molecular weight*

keratosulphate, since lower molecular weight keratosulphate is expected to have a lower critical electrolyte concentration, and this would react similarly to chondroitin sulphate in this system (Laurent and Scott, 1964). This factor may also contribute to the apparent disagreement between the results of Szirmai (1963) and those presented here.

In a new technique there are bound to be imponderables. Alcian blue is not the simplest dye with which to work; it is difficult to quantitate (Scott, Quintarelli, and Dellovo, 1964) and it may be subject to difficulties in penetration because of its considerable molecular size. The absence of staining is therefore not a reliable indication of the absence of substrate. It is considered that, in the present study, the dye is being used within its limitations, particularly in that a negative staining reaction is taken into account only in areas in which positive staining is obtained at lower salt concentrations. It will be of great interest to see if other, smaller mono-basic dyes give the same overall pattern. In this field it is not possible to dissociate the method of fixation of the tissue from the results obtained by staining. Alcian blue appears to be susceptible to "fixation artefacts" (Scott and Dorling, unpublished).

Nevertheless, it may be said that, in the present study, staining with AB —0.9M MgCl₂ in costal cartilage from infancy to old age parallels quantitative results on keratosulphate concentration (Kaplan and Meyer, 1959). On the basis of somewhat analogous reasoning—an increasing amount of staining with ageing—Conklin (1963) suggests that Bismark brown may selectively demonstrate the localization of keratosulphate. The results given here cannot confirm the selectivity of this technique, although without doubt the dye is bound by acidic groups.

The distribution of keratosulphate in the interterritorial matrix of every sample of ageing cartilage poses interesting problems. The present investigation provides no evidence for a path of secretion from the cell to that site in the matrix, yet an extracellular synthesis seems to be unacceptable; the question thus arises as to the mechanism by which the keratosulphate is deposited interterritorially. Possibly the slow turnover rate of keratosulphate compared with that of chondroitin sulphate as described in rabbit cartilage (Davidson, Woodhall, and Baxley, 1962) may be associated with its interterritorial localization. However, when the very definite territorial localization in the central region of ageing costal cartilage is taken into account, other factors must be considered. Age *per se* would not appear to influence the localization, since the cells of articular cartilage and of the middle of the costal cartilage are presumably of the same age. However, although no work has been done on this point, it seems reasonable to believe that the two regions may differ in the efficiency of their nutrition, the central region of costal cartilage being the poorer in this respect. At least in terms of distance, the cells of the latter are further away from a nutrient fluid, in this case the perichondrial capillary blood, than are all except perhaps the deepest cells of articular cartilage, assuming that the synovial fluid forms the principal if not the sole source of nutrition of this tissue (Brower, Akahoshi, and Orlic, 1962). It is possible on theoretical grounds that the availability of diphosphopyridine nucleotide (Coenzyme I) in the oxidized form could influence the relative proportions of hexose and hexuronic acid in any given population of polysaccharide molecules. On this hypothesis, a higher keratosulphate/chondroitin sulphate ratio might be expected where nutritional conditions are poorer, as suggested by Stockwell (1963).

The conclusion that chondroitin sulphate is usually present in the territorial area of young hyaline cartilage, whereas keratosulphate is characteristically present in the interterritorial area, must be of physio-

logical significance. The concentration of chondromucoprotein in the territorial areas should be high, since much of the total cartilage chondroitin sulphate appears to be there, judging by staining intensities. It is a property of concentrated solutions of highly polymerized glycosaminoglycans that they exclude from the greater part of their volume other high molecular weight material and all large particles (Laurent, 1964). This property may account for the interterritorial distribution of large fibres, and for their conspicuous absence from the territories (Galjaard, 1962, *inter alia*). This mutual exclusion provides roughly spherical volumes of concentrated glycosaminoglycan solution which may lose and regain water in a reversible fashion, to and from the interterritorial area, in response to cycles of pressure and relaxation, somewhat analogously to the operation of a telescopic shock absorber. The role of concentrated solutions of chondroitin sulphate and keratosulphate in producing swelling pressure (in the cornea) has been elegantly demonstrated by Hedbys (1961). Furthermore, the apparent replacement of chondroitin sulphate by keratosulphate in the territorial areas of the central region of ageing costal cartilage might result in a change in the properties of the "shock-absorber".

There is now good evidence that acid glycosaminoglycans are not affected by the PA-S reaction (Pearse, 1960). Thus Schiff-positive material in cartilage matrix is most likely to be glycoprotein.

From the results obtained with the PA-S technique it would appear that glycoprotein and keratosulphate are often similarly localized in the matrix and that an increase of both occurs with age.

Summary

The use of Alcian blue at a pH near neutrality and incorporating different concentrations of magnesium chloride in the staining medium, provides a method for the differentiation of the glycosaminoglycans. In cartilage, both chondroitin sulphate and keratosulphate are stained using a salt concentration of 0.4M; but only high molecular weight keratosulphate is detected using a concentration of 0.9M magnesium chloride.

Using this technique, keratosulphate has been localized in the matrix of costal and articular cartilage from ten human subjects aged 1½ to 78 years.

In infant cartilage there is a negligible staining reaction for keratosulphate. From the second decade onwards, keratosulphate is localized in the interterritorial matrix. In the central region of mature costal cartilage, however, there is an intense staining reaction for keratosulphate in the territorial

matrix, and only a weak reaction interterritorially. A similar territorial predominance is seen in the deeper parts of articular cartilage next to the calcified layer, from about the beginning of the fourth decade.

The results of the other techniques employed agree with those of earlier workers, and provide supporting evidence for the conclusions regarding the localization of keratosulphate.

The problems relating to the distribution of keratosulphate shown in this work are discussed. It is suggested that the territorial distribution of this substance in the deepest parts of both types of cartilage may be related to the efficiency of nutrition of the cells in these areas as much as to their age. The effect of these age changes on the physical properties of the tissue as a whole are discussed in the light of the principle of "excluded volume".

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Observations sur le contenu en glycosaminoglycan acide (mucopolysaccharide) de la matrice du cartilage vieillissant

RÉSUMÉ

L'emploi du bleu Alcian à un pH presque neutre et de différentes concentrations de chlorure de magnésium dans le milieu colorant offre une méthode pour différencier les glycosaminoglycans. Dans le cartilage, le sulfate de chondroïtine de même que le kératosulfate prend la coloration avec le sel en concentration de 0,4M, mais pour le kératosulfate au poids moléculaire élevé il en faut une concentration de chlorure de magnésium de 0,9M.

Avec ce procédé on a localisé le kératosulfate dans la matrice du cartilage costal et articulaire de dix sujets humains âgés de 1½ à 78 ans.

Dans le cartilage infantile la réaction colorée du kératosulfate est négligeable. À partir de l'âge de 10 ans, le kératosulfate se localise dans la matrice interterritoriale. Dans la région centrale du cartilage costal mûr la réaction colorée pour le kératosulfate est intense dans la matrice territoriale, mais très faible dans l'interterritoriale. Une prépondérance territoriale similaire s'observe dans les parties plus profondes du cartilage articulaire près de la couche calcifiée à partir de l'âge de 30 ans.

Les résultats avec d'autres procédés s'accordent avec ceux des autres auteurs et viennent à l'appui des conclusions concernant la localisation du kératosulfate.

On discute les problèmes concernant la distribution du kératosulfate démontrée dans ce travail. On pense que la distribution territoriale de cette substance dans les parties les plus profondes des deux types de cartilage peut se rapporter autant à l'efficacité de la nutrition des cellules dans ces régions qu'à leur âge. On discute l'effet de ces altérations de vieillissement sur les propriétés physiques du tissu entier, à la lumière du principe de "volume exclu".

Observaciones sobre el contenido de glicosaminoglicano ácido (mucopolisacárido) en la matriz del cartilago envejecido

SUMARIO

El empleo del azul Alcian a un pH casi neutro y de diferentes concentraciones de cloruro de magnesio en el medio colorante ofrece un método para diferenciar los glicosaminoglicanos. En el cartilago el sulfato de condroitina así como el queratosulfato toman la coloración en concentración salina de 0,4M, pero sólo el queratosulfato de peso molecular alto se puede evidenciar con una concentración de cloruro de magnesio de 0,9M.

Con este procedimiento el queratosulfato fué localizado en la matriz del cartilago costal y articular de diez sujetos humanos de edades de 1½ a 78 años.

En el cartilago infantil la reacción colorada es despreciable. Desde el segundo decenio el queratosulfato se localiza en la matriz interterritorial. En la región

central del cartílago costal maduro la reacción colorada del queratosulfato es intensa en la matriz territorial pero muy débil en la interterritorial. Un predominio territorial similar se ve en las partes más profundas del cartílago articular cerca de la capa calcificada desde el principio del cuarto decenio.

Los resultados con otros procedimientos se acuerdan con los de otros autores y favorecen las conclusiones respecto a la localización del queratosulfato.

Se discuten los problemas respecto a la distribución, evidenciada en este trabajo, de queratosulfato. Se sugiere que la distribución territorial de esta substancia en las partes más profundas de ambos tipos de cartílago pueden relacionarse tanto a la eficacia de la nutrición de las células como a la edad de estas. Se discute el efecto de estas alteraciones de envejecimiento sobre las propiedades físicas del tejido entero a la luz del principio de "volumen excluido".