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The Effect of Hyaluronic Acid on Proteoglycan Synthesis and Secretion by Chondrocytes of Adult Cartilage [and Discussion]

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INFLUENCE OF THE CELLS ON THE PERICELLULAR ENVIRONMENT

The effect of hyaluronic acid on proteoglycan synthesis and secretion by chondrocytes of adult cartilage

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[Plate 5]

The chondrocyte is a specialized cell that synthesizes proteoglycans of a type found only in cartilage and nucleus pulposus. These proteoglycans are distinct in forming multiple aggregates of unique structure in which hyaluronic acid provides a central chain to which many proteoglycan molecules are bound at one end only.

Chondrocytes were isolated from adult cartilage and used in suspension culture to test the effect of compounds in the medium on the synthesis of proteoglycans. Hyaluronic acid alone, among a number of compounds extracted from or analogous to those in cartilage, reduced the incorporation of [^{35}S]sulphate into macromolecular material. Oligosaccharides of hyaluronic acid of the size of decasaccharides and above also had this effect but hyaluronic acid already bound to proteoglycan did not.

The proportion of total labelled material associated with the cells increased at the expense of that in the medium. Treatment of the cells with trypsin abolished the effect of hyaluronic acid but treatment with chondroitinase did not. It is suggested that hyaluronic acid interacts with proteoglycans at the cell surface by a specific mechanism similar to that involved in proteoglycan aggregation, as a result of which the secretion and synthesis of proteoglycans is reduced.

Introduction

Cartilage is a highly specialized tissue whose main function is to withstand compressive mechanical forces. It is the richest source of the proteoglycans, which are very large complex polyanionic molecules that contain a number of glycosaminoglycan chains attached to a polypeptide core or backbone. There are several varieties of such molecules in connective tissue; those in cartilage, which have been studied the most contain chondroitin sulphate and lesser amounts of keratan sulphate attached to a protein core which comprises only about 10 % of the mass of the molecule.

A large proportion of the proteoglycans of cartilage can be extracted with 4 M guanidinium chloride (Sajdera & Hascall 1969) and consist of both aggregated and non-aggregated molecules (Hascall & Sajdera 1969; Rosenberg, Pal, Beale & Schubert 1970; Tsiganos, Hardingham & Muir 1971; Mashburn & Hoffman 1971). The formation of such multiple aggregates of enormous molecular size appears to be exclusive to proteoglycans of cartilage (and also of nucleus pulposus (Tsiganos & Muir 1973)). Equilibrium density gradient centrifugation of proteoglycans from bovine nasal septum in the presence of 4 M guanidinium chloride was shown to dissociate aggregates, when a protein-rich fraction separated from the majority of proteoglycans at the top of the gradient (Hascall & Sajdera 1969). This fraction was found to be

necessary for the reassociation of proteoglycans into aggregates. When a similar fractionation was applied to proteoglycans from pig laryngeal cartilage (Tsiganos *et al.* 1971), the protein-rich fraction at the top of the gradient, although binding to proteoglycans, did not promote aggregation (Tsiganos, Hardingham & Muir 1972). However, a fraction of intermediate density was found to interact with proteoglycans to produce an increase in molecular size on gel chromatography (Tsiganos *et al.* 1972). Comparable fractions of proteoglycans from other types of cartilage produced the same effect. The component of intermediate buoyant density was isolated and identified as hyaluronic acid (Hardingham & Muir 1973*a*, 1974). The interaction of proteoglycans with hyaluronic acid is highly specific and does not occur with even close analogues of hyaluronic acid such as chondroitin, i.e. chemically desulphated chondroitin sulphate (Hascall & Heinegård 1974*b*), but is prevented by oligosaccharides of hyaluronic acid of the size of decasaccharides and above which compete with hyaluronic acid (Hardingham & Muir 1973*b*; Hascall & Heinegård 1974*b*). Gregory (1973) extending the work of Hascall & Sajdera (1969) has shown that two different components were necessary for the formation of proteoglycan aggregates, one of which had a buoyant density similar to that of hyaluronic acid. The identification of hyaluronic acid in proteoglycan aggregates (Hardingham & Muir 1973*a*, 1974; Hascall & Heinegård 1974*a*) and the characteristics of the interaction with proteoglycans (Hardingham & Muir 1972) show that in the formation of aggregates, a large number of proteoglycan molecules bind to each hyaluronic acid chain and that each proteoglycan molecule possesses only a single binding site.

Using viscometry and gel chromatography to demonstrate interaction, it was shown that at a mass ratio of hyaluronic acid/proteoglycan of about 1:100, the maximum number of proteoglycan molecules were bound to each hyaluronic acid chain (Hardingham & Muir 1972); the stability of this unique structure being comparable with that of an enzyme–substrate complex (Hardingham & Muir 1973*b*).

Isotope studies have shown that proteoglycans are turned over continuously in all types of cartilage, while depletion of the chondroitin sulphate from the matrix of chick cartilage in organ culture by the action of hyaluronidase, stimulated the synthesis of proteoglycans about fivefold compared with the control rate (Hardingham, Fitton Jackson & Muir 1972). This suggests that chondrocytes are responsive to the state of the matrix that surrounds them. The possibility has therefore been investigated that a constituent of the matrix may control proteoglycan synthesis in some way.

Because joint diseases mainly affect adults, cells from adult cartilage were used in this study, and in order to test the effects of added compounds on the cells, they had first to be isolated from the cartilage matrix. These isolated cells survived in suspension for at least 3 weeks without dividing, and thus resembled cells in adult cartilage which do not normally divide. The test consisted of measuring the incorporation of [^{35}S]sulphate into macromolecular material by the isolated cells, and the compounds tested were extracted from or resembled those in cartilage. The earlier observation that hyaluronic acid in very low concentration reduced this incorporation (Wiebkin & Muir 1973*a*, *b*) is examined further here.

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Materials and methods

The media, chemicals and enzymes used were those previously described (Wiebkin & Muir 1973*a*). Hyaluronic acid labelled biosynthetically with [^{14}C]glucosamine was a gift of Dr J. Gallagher; proteoglycan aggregates, dissociated proteoglycans and proteoglycan–hyaluronic acid complex (mass ratio 75:1) was generously provided by Dr T. E. Hardingham. The tissue commonly used was the thyroid cartilage of bacon pigs obtained fresh from the slaughter house. Occasionally articular cartilage from these and older pigs was used, as was articular cartilage of adult rabbits. No differences were found between the cells from different types of cartilage.

Isolation of cells

The cartilage was scraped free of soft connective tissue and perichondrium, and chopped into small pieces as described (Wiebkin & Muir 1973*b*), the cartilage from each animal being treated separately. The cartilage was placed in chambers with a nylon mesh bottom which allowed cells that had been freed of their matrix to pass through (Green 1971). The tissue was first incubated for 10 min at 37 °C with 5–7 ml of a solution of testicular hyaluronidase (EC 3.2.1.35) containing 500 units/ml in basal salt solution. After washing, the tissue was incubated for 30 min at 37 °C with 5–7 ml of a solution of trypsin (EC 3.4.4.4) containing 258 units/ml in basal salt solution. Finally, after washing, the tissue was digested by stirring for 18 h at 37 °C with 5–10 ml of a solution containing 125–200 units/ml of bacterial collagenase (EC 3.4.4.19) in Leibovitz L-15 medium enriched with 10% foetal calf serum to which streptomycin (100 μg/ml) and penicillin (100 i.u./ml) were added. The cells that had passed through the mesh were freed of debris by centrifugation in a discontinuous Ficoll gradient, washed and resuspended in enriched L-15 medium (Wiebkin & Muir 1973*b*). The exclusion of Trypan Blue and the incorporation of [^{35}S]sulphate indicated that the cells were viable for at least three weeks, the medium being changed every other day. No mitotic figures were observed and there was no incorporation of [^{3}H]thymidine (Wiebkin & Muir 1973*b*).

[^{35}S]*sulphate incorporation*

The cells were washed and transferred to Tyrode's solution which contained no serum. Cell suspensions were divided into aliquots containing 5×10^5 cells/ml and pairs selected appropriately as controls and experimental samples, each pair being obtained from one animal or culture, the experiments being done in triplicate.

Test solutions were prepared by appropriate dilution of hyaluronic acid or other test compound with Tyrode's solution to which 0.25–0.5 mCi of [^{35}S]sulphate was added. The cell suspensions were incubated for 2 h at 37 °C after which cells and medium were separated and ^{35}S-labelled material isolated from each and the radioactivity measured as described (Wiebkin & Muir 1973*b*).

In those experiments where the radioactivity of the material associated with the cell surface was also measured, the cells were separated from the medium and washed. The cells were then digested for 30 min at 37 °C in 2 ml of basal salt solution containing 258 units/ml of trypsin and 18.5 units/ml of deoxyribonuclease (EC 3.1.4.5.) to solubilize the outer 'calyx' of the cell (Kraemer 1971). The radioactivity of CPC precipitable material in the original medium and in the supernatant after trypsin treatment was measured. Non-radioactive trypsinized cells were prepared in the same way.

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The uptake by the cells of [^{14}C]hyaluronic acid was determined by incubating the cells for varying times up to 2 h with [^{14}C]hyaluronic acid in the medium. To test the effect of this labelled hyaluronic acid on the incorporation of [^{35}S]sulphate by the cells, double labelling experiments were carried out, in parallel with each respective single label experiment to enable the contribution of each isotope to be calculated. The cells and medium were separated and the cells washed and trypsinized. The radioactivity in the medium and associated with the cells and the cell surface was then measured.

Results

The isolated cells were maintained in suspension for a minimum of 4 days to allow them to recover before being used in the standard test, by which time electron micrographs showed them to be healthy with well defined endoplasmic reticulum and Golgi apparatus (figure 1, plate 5). The incorporation of [^{35}S]sulphate into macromolecular material during 2 h took place at the same rate whether the cells were in contact in the form of a pellet or in suspension, but there was no incorporation if the cells had been killed by freezing and thawing. Throughout the experiments the reproducibility in the standard test showed a standard deviation of 7 %.

In earlier experiments, various compounds known to be present in cartilage and their analogues had been tested for effects on [^{35}S]sulphate incorporation (Wiebkin & Muir 1973*a*). These included cartilage proteoglycans, free chondroitin 4-sulphate, and dermatan sulphate, cartilage and egg-white lysozyme and hyaluronic acid. With the exception of hyaluronic acid, none of these compounds had significant effects in low concentrations, whereas hyaluronic acid reduced the incorporation of [^{35}S]sulphate in the test system by 25 % at 5×10^{-3} μg/ml and by 50 % at 0.1 μg/ml (Wiebkin & Muir 1973*b*). The addition of hyaluronic acid to the medium invariably reduced the incorporation of [^{35}S]sulphate but this never exceeded 50 % even at much higher concentrations. The inhibitory effect of hyaluronic acid appeared to be restricted to chondrocytes as it had no effect on skin fibroblasts or synovial cells even at concentrations as high as 10 μg/ml (Wiebkin & Muir 1973*b*).

In parallel with the reduction in [^{35}S]sulphate incorporation, the proportion of the total radioactivity associated with the cells increased considerably (Wiebkin & Muir 1973*a*, *b*). The proportion bound to the cell surface was shown by trypsinization of the cells. Table 1 shows that this fraction did not change significantly in the presence of hyaluronic acid; whereas the proportion of the total radioactivity within the cells increased at the expense of the proportion in the medium. This effect diminished with diminishing concentrations of hyaluronic acid in the medium. When 0.1 μg/ml was present, the total incorporation was reduced to 67 % of the control value but the proportion within the cells almost doubled from about a quarter to about half, while the proportion of the total radioactivity in the medium decreased from two thirds to less than half (table 1). No broken cells were observed on microscopy after the mild trypsinization.

Using pre-labelled cells, preliminary results indicate that the proportion of ^{35}S-labelled material entering the medium during 2 h of incubation was reduced by about a quarter when 0.1 μg/ml of hyaluronic acid was present in the medium.

The inhibitory effect of hyaluronic acid on [^{35}S]sulphate incorporation appeared to be equally specific as the interaction of hyaluronic acid with proteoglycan. Even close analogues of hyaluronic acid such as chondroitin (chemically desulphated chondroitin sulphate) had no

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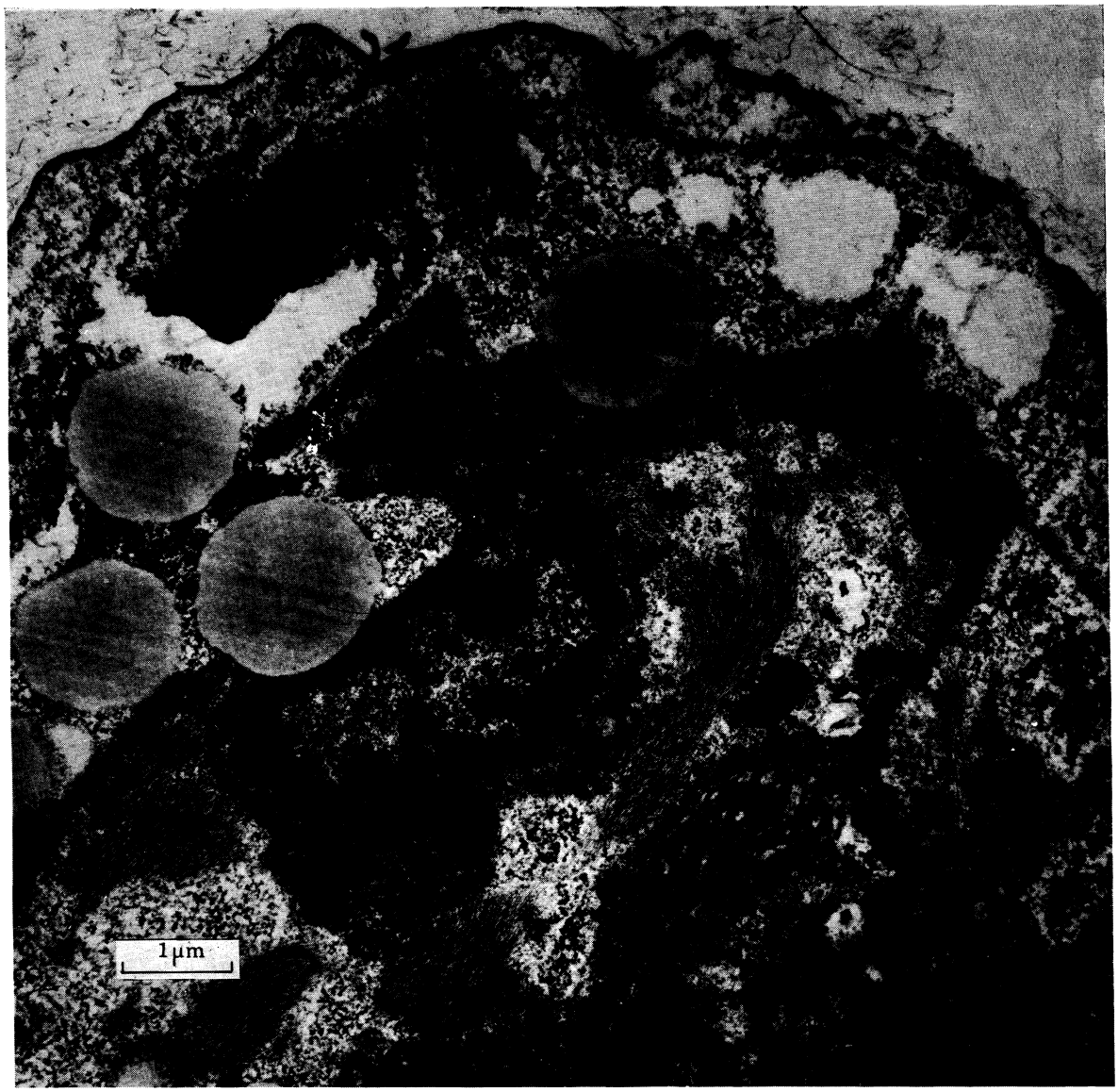

FIGURE 1. Electron micrograph of chondrocyte, enzymatically isolated from adult pig laryngeal cartilage and cultured for 4 days. Fixed with glutaraldehyde followed by osmium tetroxide; stained with uranyl acetate and lead citrate.

(*Facing p.* 286)

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effect (table 2). Chondroitin which contains galactosamine in place of glucosamine possesses the same glycosidic linkages as hyaluronic acid and differs only in the configuration of the hydroxyl group on C_4 of the hexosamine residues.

Hyaluronic acid already combined with proteoglycan in a complex or in natural aggregates had no inhibitory effect whereas the same amount of free hyaluronic acid markedly reduced [^{35}S]sulphate incorporation. Free proteoglycan when added in the same amount as that combined with hyaluronic acid in the complex had no effect either (table 3).

TABLE 1. INCORPORATION OF [^{35}S]SULPHATE INTO MACROMOLECULAR MATERIAL OF CELLS, CELL SURFACE AND MEDIUM IN THE PRESENCE OF HYALURONIC ACID (HA)

	detected radioactivity per 10^6 cells							
[HA] μg/ml ...	0.1		0.01		0.001		control (no HA)	
		% of total		% of total		% of total		% of total
medium	5875	50	6875	54	8446	57	11580	67
cells	5000	43	4750	38	5092	35	4555	27
cell surface	812	7	1037	8	1145	8	1087	6
total detected radioactivity	11687		12662		14683		17275	(=100)
percentage of control radioactivity	67.7		73.3		85		100	

TABLE 2. THE INFLUENCE OF FREE HYALURONIC ACID AND PROTEOGLYCAN, OR COMBINED IN A COMPLEX ON [^{35}S]SULPHATE INCORPORATION INTO MACROMOLECULAR MATERIALS

additive	HA		HA–PG complex		PG		control
μg/ml	0.1	0.01	7.4	0.74	7.3	0.73	0.0
HA content	0.1	0.01	0.1	0.01	0.0	0.00	0·0
detected radioactivity per 10^6 cells	10200	11200	18900	16200	19300	15300	15000 (av)

TABLE 3. THE INFLUENCE OF CHONDROITIN SULPHATE AND CHONDROITIN† ON [^{35}S]SULPHATE INCORPORATION INTO MACROMOLECULAR MATERIAL

additive	amount μg/ml	total incorporation: medium and cells (% of controls)	proportion of total detected radioactivity associated with cells (%)
chondroitin sulphate	20.0	100.0	39.0
	10.0	110.0	37.0
chondroitin†	20.0	98.0	35.0
	10.0	107.0	37.0
	1.0	102.0	32.0
none (control)	0.0	100.0	33.0

† Chemically desulphated chondroitin sulphate.

Oligosaccharides of hyaluronic acid that were large enough to be excluded from Sephadex G 25, also reduced [^{35}S]sulphate incorporation when present in the culture medium at concentrations equivalent to 0.01 μg/ml and above, whereas smaller oligosaccharides had no effect (table 4).

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[^{14}C]hyaluronic acid was taken up by the cells, the uptake being complete after 60 min of incubation. Most of the label taken up by the cells could be removed by mild trypsinization of the cells.

The inhibitory effect of hyaluronic acid on [^{35}S]sulphate incorporation was entirely abolished by prior trypsinization of the cells, but not when the cells were first digested with chondroitinase ABC (figure 2), even though about the same amount of uronic acid was removed from the cells by both treatments. In each case the tests were performed 6 h after the enzyme treatments.

Trypsin treatment reduced the total incorporation of [^{35}S]sulphate considerably but the cells quickly recovered their synthetic capacity during 24 h, by which time the inhibitory effect of hyaluronic acid was restored (table 5).

TABLE 4. THE INFLUENCE OF OLIGOSACCHARIDES OF HYALURONIC ACID SEPARATED BY GEL CHROMATOGRAPHY (SEPHADEX G 50 and G 25) ON [^{35}S]SULPHATE INCORPORATION INTO MACROMOLECULAR MATERIAL

oligosaccharide fractions†	[uronic acid] µg/ml	total incorporation (% of controls)
A	0.1	50 ± 6
	0.01	74 ± 4
	0.001	104 ± 15
B	0.1	54 ± 2
	0.01	60 ± 25
	0.001	76 ± 8
C	0.1	111 ± 9
	0.01	93 ± 9
	0.001	98 ± 28
E	0.1	86 ± 6
	0.01	115 ± 20
	0.001	117 ± 3
control		100 ± 15

† Oligosaccharides were prepared from hyaluronic acid after partial digestion with testicular hyaluronidase by chromatography on Sephadex G 50 to remove undigested material. Fractions A–E in order of elution from Sephadex G 25: (A and B excluded; C and E retarded).

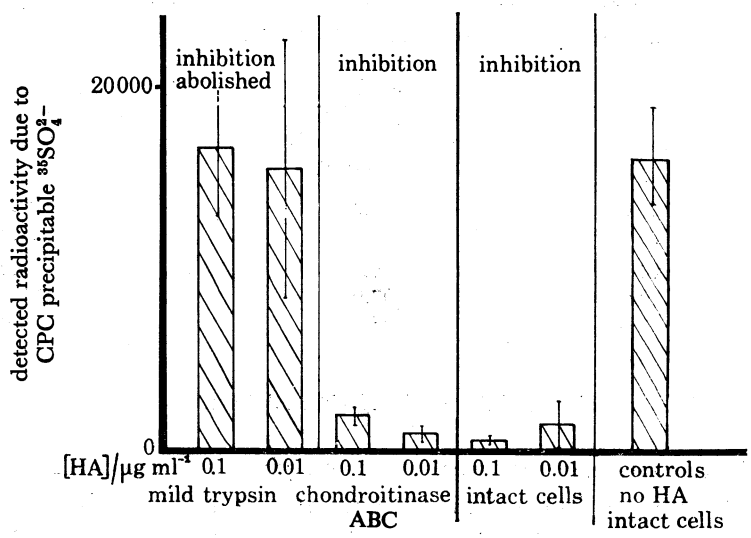

FIGURE 2. Treatment of cells with trypsin or chondroitinase ABC; the effect 6 h later on the inhibition of [^{35}S]sulphate incorporation by hyaluronic acid (range shown by bars).

TABLE 5. THE INFLUENCE OF HYALURONIC ACID ON [^{35}S]SULPHATE INCORPORATION INTO MACROMOLECULAR MATERIAL BY CHONDROCYTES AFTER RECOVERY FROM MILD TRYPSIN TREATMENT

time after trypsin treatment	detected radioactivity per 10^6 cells: concentration of hyaluronic acid in medium/μg ml^{-1} 0.1	0.01	control (no HA) 0
5 min	7500	4300	4800
6 h	17000	15000	11200
18 h	13300	11000	19300
untreated cells	12000	11800	17400

DISCUSSION

The 2 h incorporation of [^{35}S]sulphate into CPC precipitable material was an arbitrary test. It was chosen because it was long enough to enable a steady-state to be established and short enough to avoid side effects which might result from exhaustion of nutrients since the cells were placed in Tyrode's solution during the test with no additives other than [^{35}S]sulphate. The time was probably too short for products secreted by the cells to accumulate in the medium and perhaps affect the behaviour of the cells. Tyrode's solution was used rather than nutrient medium to avoid any interaction of added hyaluronic acid with serum proteins which could interfere with its action, and also because Tyrode's solution contained no inorganic sulphate to dilute the isotopic sulphate enabling higher specific incorporation to be achieved.

The interaction of proteoglycans with hyaluronic acid is entirely specific to hyaluronic acid and since the inhibitory effect on the incorporation of [^{35}S]sulphate into CPC precipitable material was equally specific to hyaluronic acid, it seemed possible that it was effected through interaction of hyaluronic acid with proteoglycans on the cell surface of the chondrocytes.

This conclusion is supported by the following points:

(1) Proteoglycans that undergo aggregation and hence are able to interact with hyaluronic acid occur in cartilage, but not in other types of connective tissue such as skin. Hyaluronic acid had no effect on skin fibroblasts or synovial cells.

(2) The concentrations of hyaluronic acid that reduced [^{35}S]sulphate incorporation were low and showed a low threshold, above which there was no additional effect. This result is in keeping with the stoichiometry of the interaction of hyaluronic acid with proteoglycans (Hardingham & Muir 1972, 1974) where a large number of proteoglycan molecules are bound to a single hyaluronic acid chain, each proteoglycan molecule having only one binding site.

(3) Oligosaccharides of hyaluronic acid of the size of decasaccharides and above interact with proteoglycans in competition with hyaluronic acid (Hardingham & Muir 1973*b*; Hascall & Heinegård 1974*b*). Likewise, fractions of hyaluronidase digests of hyaluronic acid, that contained larger oligosaccharides reduced [^{35}S]sulphate incorporation, whereas fractions that contained only small oligosaccharides retarded by Sephadex G 25 did not.

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(4) Proteoglycans, from which the condroitin sulphate chains have been removed by digestion with chondroitinase ABC, are still able to interact with hyaluronic acid (Hascall & Heinegård 1974*b*), but not after exhaustive proteolysis with trypsin (Hardingham, unpublished results). In keeping with this, treatment of the cells with trypsin but not with chondroitinase abolished the inhibitory effect of hyaluronic acid.

(5) Since the cells regained their response to hyaluronic acid 24 h after trypsin treatment, it would seem that a component which interacts with hyaluronic acid had been stripped off the cell surface by trypsinization, which subsequently was resynthesized.

Another effect of hyaluronic acid was to increase the proportion of labelled material within the cell apparently by reducing secretion as shown by preliminary results on the effect of hyaluronic acid on prelabelled cells. This suggests that intracellular accummulation reduced the total synthesis of proteoglycan. Whether this effect is purely physical due to the formation of aggregates at the cell surface or is of a more specific nature, cannot be decided yet. The fact that oligosaccharides of the size of decasaccharides (molecular mass 2000) also had an inhibitory effect suggests that it may not be purely physical in nature since oligosaccharides to which two proteoglycans can bind were calculated to be much larger than decasaccharides, i.e. with molecular masses above 10000 (Hardingham & Muir 1973*b*).

Some of the proteoglycans in cartilage are unable to interact with hyaluronic acid (Hardingham & Muir 1974). These presumably would be unaffected by hyaluronic acid and would be secreted normally. In addition, preliminary results show that the inhibitory effect of hyaluronic acid faded gradually when the cells were placed in medium that contained no hyaluronic acid, presumably because material on the cell surface was continuously sloughed off since the cells were in suspension. Both these factors might explain why the incorporation of [^{35}S]sulphate was never reduced by more than half.

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Discussion

D. H. Leaback (*Institute of Orthopaedics, Stanmore, Middlesex*). Did I understand you to say in your talk that you had estimates of the molecular mass of the hyaluronate in cartilage? If so how did you carry out the estimations?

H. Muir. We have an approximate idea of the molecular mass of the hyaluronic acid in cartilage from its elution profile from Sepharose 2B in comparison with the profile of hyaluronic acid prepared from other tissues, whose molecular mass was estimated by viscometry or by light scattering. The hyaluronic acid in cartilage was very polydisperse and of much lower average molecular mass than the specimen from cock's comb (gift of Professor T. Laurent).

D. H. Leaback. I think you said in your talk that you did not know the origin of the hyaluronate that occurs in cartilage. Would you not agree that it is difficult to imagine how a large molecule like hyaluronate could get into the matrix unless it is synthesized by the cartilage cells?

H. Muir. Hyaluronic acid is most probably synthesized by chondrocytes.

R. A. Stockwell. Is there any evidence for hyaluronidase activity in cartilage?

H. Muir. So far as I know, there is no evidence for hyaluronidase activity in cartilage. However, it is not an easy enzyme to detect and therefore one should not be too dogmatic in stating that there is none.

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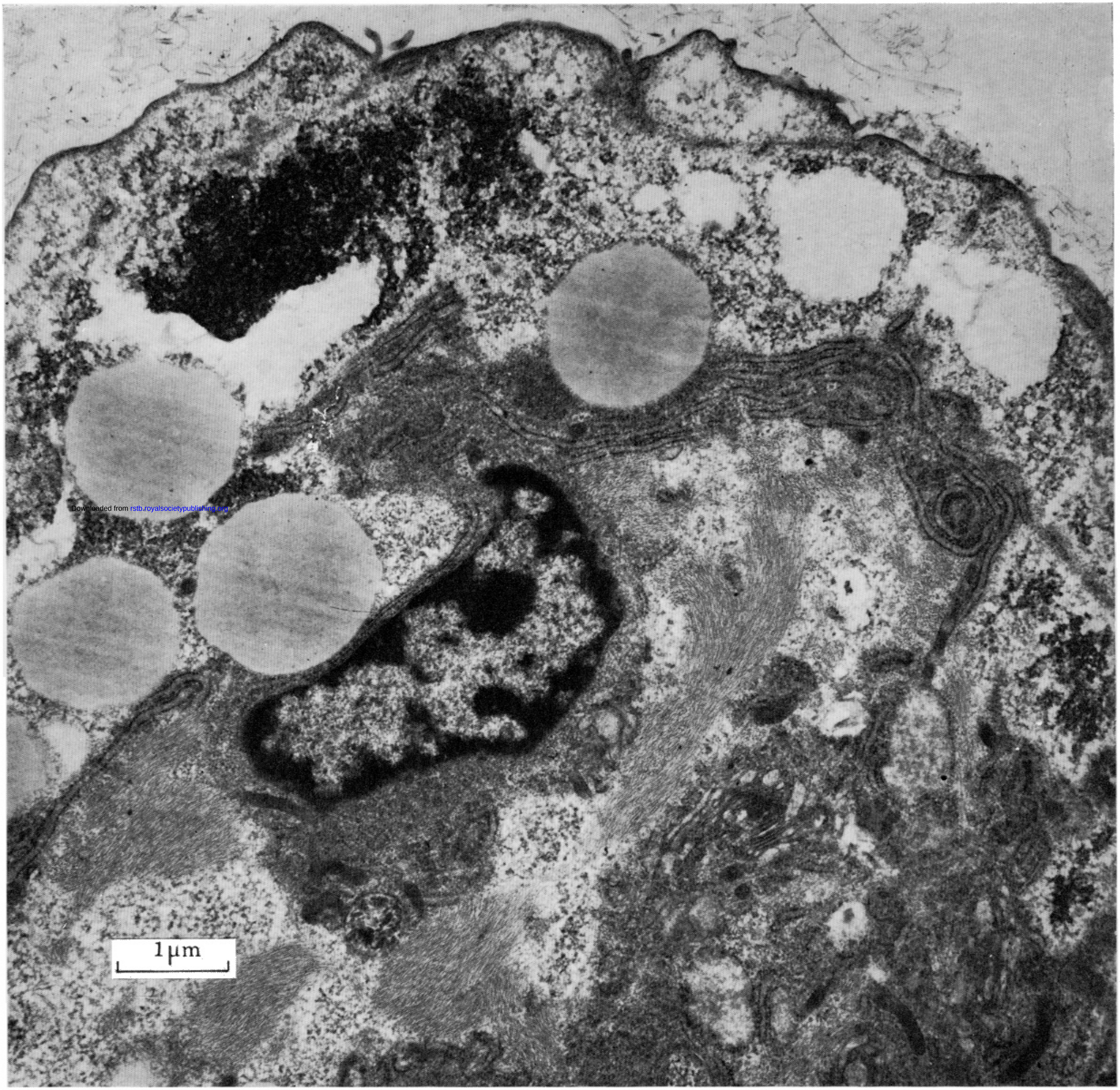

FIGURE 1. Electron micrograph of chondrocyte, enzymatically isolated from adult pig laryngeal cartilage and cultured for 4 days. Fixed with glutaraldehyde followed by osmium tetroxide; stained with uranyl acetate and lead citrate.