phosphorus content the latter tissue contains 50 % more creatine and phosphocreatine than the comparable human material.

Table 3 shows quite clearly that in proliferative rheumatoid-villous tissue there is not only a considerable reduction in the proportion of bound creatine but also a marked drop in the levels of total creatine relative to the tissue deoxyribonucleic acid-phosphorus content. However, the massive synovial proliferation which takes place in the active-disease state is accompanied by a considerable change in subsynovial-cell types (Thomas & Dingle, 1959), and the changes observed in the ratios of creatine and phosphocreatine to deoxyribonucleic acid-phosphorus may well reflect these alterations in cell populations.

SUMMARY

1. The method of Ennor & Rosenberg (1952) for the estimation of creatine and phosphocreatine has been simplified and applied to various tissues including synovial tissue from human and rabbit.

2. Cellular disruption was brought about by a solution containing Tween 80, ethylenediamine-tetra-acetic acid, *p*-chloromercuribenzoic acid and alkali. The extraction of creatine from the sliced softer tissues was 97 %, but synovial tissue proved more resistant, with an average extraction of 87 %.

3. Quantitative recoveries of added creatine and phosphocreatine were obtained, and results for rat tissues agree with those found by other authors.

4. Human synovial membrane contained 5.0 mg. of creatine and 2.4 mg. of phosphocreatine/100 g.

of wet tissue, and normal rabbit synovial tissue contained 5.8 and 2.6 mg. respectively. The proportion of creatine bound as phosphate was roughly 22 % for each type of tissue. Rheumatoid-villous tissue contained 7.4 mg. of creatine and only 0.9 mg. of phosphocreatine/100 g. of wet tissue.

5. There was a considerable decrease of creatine (in relation to the amount of deoxyribonucleic acid) in rheumatoid-villous tissue, but this may be due to the considerable redistribution of cell types which accompanies the active disease state.

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A Structural Function of Mucopolysaccharide in Connective Tissue

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The function of hyaluronic acid in connective tissue is unknown. Studies described here show that one possible function of hyaluronic acid is a mechanical one: a combined structure of hyaluronic acid, water and collagen fibres can be made which has a definite resistance to compression.

A general investigation of the Wharton's jelly of umbilical cord was made and led to comparisons

with hyaluronic acid complex (Ogston & Stanier, 1950) of synovial fluid. Model experiments were then made in which mixtures of synovial fluid and fibres of soluble collagen were ultracentrifuged, and they showed properties consistent with the mechanical functions proposed. It is suggested that any suitable combination of water, a high polymer of open structure and fibres, can form a system resistant to temporary mechanical compression. This view, together with a summary of some of the work, has been described (Fessler, 1957).

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EXPERIMENTAL

Materials

Umbilical cords (human). These were collected as soon as possible after birth, externally cleaned and placed in dry containers at 0°. Blood-stained or grossly swollen cords were discarded. Most of the blood was gently removed from the vessels and the ends of the cords were usually tied. Umbilical cords were used within 18 hr. of birth and often much earlier.

Synovial fluids. These were collected and stored as described by Fessler, Ogston & Stanier (1954). To bovine fluids Merthiolate [sodium ethyl mercurithiosalicylate solution no. 45 (0.1%, w/v), monoethanolamine (0.1%, w/v), Boots Pure Drug Co. Ltd., Nottingham] was added to a final concentration 1:34 000 (w/v).

Soluble collagen. Bovine-hide, neutral-salt-soluble collagen was kindly donated by Dr Jerome Gross, Massachusetts General Hospital, Boston, Mass., prepared as described by Gross (1958). It was concentrated to small volume by dialysis against 25% polyvinylpyrrolidone in the high-ionic-strength buffer and redissolved by brief dialysis against 1% acetic acid and exhaustively dialysed against the high-ionic-strength buffer. The resulting highly viscous solution was partly clarified by ultracentrifuging (Spinco rotor no. 30, 30 000 rev./min., 3 hr.). The final concentration was 3.2 mg./ml.

Amniotic fluid. This was collected under sterile conditions at surgical induction of labour and was used with the corresponding umbilical cord.

Buffers. Except where stated, the buffer used was 0.2M-NaCl-7.7 mM- Na_2HPO_4 -2.3 mM- KH_2PO_4 , pH 7.3. The high-ionic-strength buffer for experiments involving soluble collagen was sodium phosphate buffer, pH 7.6, 1.0M. AnalaR reagents were used throughout. Solutions were kept saturated with toluene by addition of a small, wetted dialysis bag filled with toluene. Merthiolate was added in final concentration 1 in 34 000 (w/v).

Hyaluronidase. Lyophilized Wydase, John Wyeth and Bros. Ltd., London, was used.

Methods

Swelling of umbilical cords. Cleaned, blotted cords were tied at both ends and suspended as a loop in a measured volume of fluid, so that only the ends projected above the fluid. The covered containers were stored at 2-4° and the weight of the blotted cords was taken from time to time. Percentage swelling was expressed as: [(difference from initial weight)/(initial weight)] $\times$ 100.

Excision of Wharton's jelly. The umbilical vein and the whole cord were gently and quickly rinsed with water, blotted dry and the ends of the cord were tied. The umbilical vessels were dissected out together with their adjoining structures. The remainder was cut up with scissors, or ground in a Latapie mincer, and weighed. Care was taken throughout to avoid contamination with blood, and while the material was kept clear of gross fluid, it was occasionally moistened with water.

Extraction of Wharton's jelly. Minced material was gently stirred, avoiding frothing, with 1-5 times its weight of water or buffer at 2-4°. After several hours the mixture was ultracentrifuged (Spinco rotor no. 40, 39 000 rev./min., 20 min.). The supernatant was dialysed, if necessary,

against several changes of buffer. The precipitate could be re-extracted several times. An extract could also be made by directly ultracentrifuging pieces of Wharton's jelly obtained by blunt dissection from a swollen umbilical cord.

Concentration of synovial fluid. Bovine fluid (250 ml.) was clarified by ultracentrifuging (Spinco rotor no. 30, 25 000 rev./min., 90 min.) and dialysed against repeated changes of a 25% solution of polyvinylpyrrolidone in the buffer of high ionic strength. As concentration proceeded, transient precipitates formed and these were partly dissolved by gentle mixing. Viscous fluid (60 ml.) remained and was completely clarified by ultracentrifuging (Spinco rotor no. 30, 25 000 rev./min., 90 min.).

Preparation and analysis of mixtures of concentrated synovial fluid and soluble collagen. With care to keep all materials cold, concentrated soluble collagen solution and bovine synovial fluid were weighed into a Lustroid tube (approx. $1\frac{1}{2}$ in. $\times$ $\frac{3}{8}$ in. for Spinco rotor no. 40, with adaptor) and well stirred, and the tube was stoppered and left in the refrigerator overnight or longer. Tubes, sterile paraffin and a Spinco rotor no. 40 were kept for 3 hr. in a water bath at 37°. Tubes were topped up with the paraffin and without disturbing the gels were quickly placed in the dried rotor and transferred to an ultracentrifuge at room temperature (23-29°). The centrifuge was evacuated and by manual control linearly accelerated to 30 000 rev./min. in 5 min., kept at this speed for 13 min. and stopped in the usual way with the brake. Control experiments showed that the temperature of solutions did not fall below 34°, at most, during these operations. Aqueous supernatants were withdrawn and stored in stoppered tubes. Most of the paraffin was removed, the precipitates were cleanly spooned out of the tubes and quickly blotted between hard filter paper and weighed. Tubes with only concentrated collagen or synovial fluid were run in parallel, and their supernatants were combined in the same proportions by weight as the mixtures used to make collagen-synovial fluid gels. Flow times were determined for 0.5 ml. samples of final solutions in an Ostwald viscometer (buffer time 61 sec.) at 30°. About 50 units (one-third of a vial) of freeze-dried Wydase were added, mixed, and the stoppered viscometer was incubated. Flow times were measured at intervals; flow times decreased only during the first hour of incubation, and were unaltered by incubation if no hyaluronic acid was present. The concentration of hyaluronic acid complex in stock concentrated synovial fluid was measured by the method of mucin clots. A calibration graph for determining hyaluronic acid content was made by measuring the relative viscosities, before and after the action of hyaluronidase, of a set of dilutions of stock concentrated synovial fluid. Degraded bovine synovial fluid was made by incubating 3 ml. of concentrated fluid with 40 units of Wydase, thereby reducing the relative viscosity from 142 to 1.6. The control fluid was incubated in parallel with heated enzyme (140° for 45 min.); its final relative viscosity was 91.

Hyaluronic acid complex concentration. This was measured by the method of mucin-clot weight of Ogston & Stanier (1950). To ensure that sufficient protein was present for mucin-clot formation, a protein solution was added in excess when necessary. This solution was prepared from bovine synovial fluid by removing hyaluronic acid on a sintered-glass filter (Ogston & Stanier, 1950) and dialysing the filtrate against distilled water. Ogston & Stanier (1952)

found that 45% by weight of a bovine mucin clot is hyaluronic acid complex. The same composition was assumed for mucin clots of umbilical cord extract, as the hyaluronic acid complex could not be isolated by the method of Ogston & Stanier (1950) on a glass filter of 1 μ pore size.

Collagen concentration. This was measured as hydroxyproline by the method of Gross (1958).

Ultracentrifuging. For preparation the Spinco model L ultracentrifuge was used; for analysis the Svedberg oil ultracentrifuge, installed in Oxford, was used at 25–28° as described by Cecil & Ogston (1948); sedimentation coefficients were calculated by the method of the same authors and corrected to water at 20°.

Refractometry. The bi-prism refractometer of Cecil & Ogston (1951) was used.

Couette viscometry. This was performed as described by Fessler *et al.* (1954).

Electron microscopy. Regions of umbilical cord distant from blood vessels and the fibrous regions associated with them were always selected. Cubical samples of about 3 mm. side were chopped with a razor blade, mildly disintegrated in a Potter–Elvehjem glass homogenizer, fixed in formalin–0.9% sodium chloride (1:99, v/v) and placed on nitrocellulose-covered grids. Alternatively, the grids were touched against the side of an incision in a cord, the sample was allowed to dry and then fixed in 1% phosphotungstic acid. After several washes in water, many samples were shadowed with gold–palladium at 30° angle.

RESULTS

Umbilical cord

Swelling of umbilical cord. Cords were immersed in various solutions and their weights recorded at intervals up to 160 hr. Most of the swelling had occurred after 100 hr. and some results are summarized in Table 1. The degree of swelling varied with tonicity and pH of the bathing fluid: amniotic fluid buffered to pH 7.4 [with expired air (approx. 4% of CO₂)] gave the least swelling. The swollen cords were rigid structures, which strikingly maintained their rigidity even if cut or split open by the swelling: most of the imbibed water was thus held throughout the matrix of the cords. Compression of pieces of swollen cord (Spinco ultracentrifuge, 30 000 rev./min., 20 min.) released the imbibed water, together with mucopolysaccharide and protein, leaving a compact but still resilient residue. Similar compression of pieces of cord which had not been swollen did not release much fluid, though this varied from cord to cord.

Extraction of Wharton's jelly. The effect of increasing time of extraction, repeated extraction and finer subdivision of the jelly than that obtained with the Latapie mincer gave the impression that the polysaccharide was mechanically occluded within the jelly. Table 2 shows that the yield of polysaccharide increased with increasing time of extraction to a plateau value after about 20 hr.

Table 1. *Swelling of umbilical cords immersed in different fluids*

A summary of results of numerous experiments is shown, the cords being weighed at intervals up to 160 hr.

Percentage swelling

$$= \frac{\text{Wt. of swollen cord} - \text{initial wt. of cord}}{\text{Initial wt. of cord}} \times 100.$$

Fluid	Percentage swelling of cord, approx. steady condition after soaking for 100 hr.
Several changes of water	170
Limited volume of water	90
Amniotic fluid (unbuffered)	49
0.067 M-Phosphate buffer, pH 7.4	32
NaCl solution (20%, w/v)	22
Amniotic fluid, buffered	13

Table 2. *Progress of extraction of Wharton's jelly with time of extraction*

Yield of polysaccharide measured as mucin clot. 1 part of minced Wharton's jelly was added to 3 parts of water and a clot made from 1 ml. of extract.

Extraction time (hr.)	Clot wt. (mg.)
2	2.81
	2.99
3½	3.21
	3.21
20	3.4
	3.31

Yield measured as relative viscosity of extract

Extraction time (hr.)	Relative viscosity
6	5.7
10	6.0
34	6.8
60	6.9

Table 3. *Effect of various treatments on the relative viscosity (η_R) of bovine synovial fluid, at different shear rates*

The relative viscosity was measured at numerous shear rates in a Couette viscometer. For intermediate shear rates the relative viscosity of the native fluid was always greater than that of a fluid exposed to any of the treatments. $\eta_0 = \eta_R$ extrapolated to zero shear rate. $\eta_{50} = \eta_R$ at 50 sec.⁻¹ shear rate.

Treatment	η_0	η_{50}
None	7.7	6.0
Freezing and breaking into fragments	7.4	5.5
Razor-blade homogenizer	6.75	5.32
None	6.4	4.0
Potter–Elvehjem homogenizer	3.1	2.34

Repeated extraction, the jelly being pressed out in the ultracentrifuge after each extraction, showed that three extractions exhausted the material which could be removed in this way. The residue was briefly homogenized in a Potter-Elvehjem homogenizer and a further extract made; it contained ample polysaccharide but no protein (by sulphosalicylic acid test). Such further subdivision could not be used to extract material for the present experiments because hyaluronic acid is degraded during such procedures. This was shown by measuring the effect on the viscosities of bovine synovial fluid of homogenizing (Potter-Elvehjem), freezing in liquid air and breaking into fragments, and subdivision in a mechanical-razor-blade homogenizer (of the Waring Blender type) (Table 3).

Electron microscopy. Fine-banded fibres, about 400 Å wide and wider, were found lying free in the matrix of Wharton's jelly; their pattern of cross-striation was such as is found in the collagen of young animals. Although the fibres were bigger and more densely placed in some regions, no evidence of any definite meshwork was seen. The fibres and background matrix were removed by digestion with collagenase plus hyaluronidase at 37°. The fibres were similarly present in the umbilical cord of a 24-week-old foetus.

Extracts of umbilical cord

Physical properties. The results are given in Table 4, and, for comparison, measurements made on human, sheep and ox synovial fluids (Fessler *et al.* 1954) are included. Molecular parameters were calculated by the treatment of Ogston (1953), assuming: partial specific volume of hyaluronic acid complex 0.69 [Ogston & Stanier (1950) deter-

mined for ox hyaluronic acids]; viscosity of solvent 10^{-2} , density of solvent 1 (properties of water at 20°). Free protein was present in the extracts; its contribution to measured relative viscosities was

Fig. 1. Comparison of anomalous viscous behaviour of extracts of human umbilical cord (●) and human synovial fluids (○) with common curve A drawn through them. η_0 , relative viscosity at zero shear rate; η_{50} , relative viscosity at 50 sec.⁻¹ shear rate. Curve B: behaviour of ox synovial fluid. Synovial fluid results are from Fessler, Ogston & Stanier (1954).

Table 4. *Physical properties of hyaluronic acids*

The sedimentation coefficients, intrinsic viscosities and molecular dimensions of hyaluronic acids are shown.						
A, Sedimentation coefficient S_{20}^0 , corrected to water at 20° and extrapolated to zero concentration (in absolute units); B, rate of change of reciprocal of corrected sedimentation coefficient with concentration (g./100 ml.);						
C, intrinsic viscosity = $\text{Lt}_{c \rightarrow 0} \left(\frac{\eta_0 - 1}{c} \right)$, where η_0 is the relative viscosity ($\eta_{\text{sol.}}/\eta_{\text{buffer}}$) extrapolated to zero shear rate;						
D, particle weight M in molecular-weight units; E, effective specific volume V' of the hydrated particle (ml./g.);						
F, ellipticity J , the axial ratio of the hydrated particle (axis of rotation/equatorial axis).						
Material and sample	A $10^{13} S_{20}^0$	B $10^{13} d(1/S_{20})/dc$	C $[\eta]_c$	D $10^{-6} M$	E V	F J
Human knee fluid*						
Wo	5.4	1.5	22	1.2	68	18
Va	5.2	1.3	36	1.3	120	30
Pe	9.5	2.3	30	3.7	220	10
Human umbilical-cord extract						
aQ	—	—	54	4.5	235	14
aL	10.0	2.55	35	4.1	274	10
aP	—	—	22	3.6	310	6
Sheep-ankle fluid*	11.5	2.6	35	5.5	320	8
Ox-ankle fluid*	14.7	3.3	55	10	520	8

* Taken from Fessler *et al.* (1954).

Table 5. *Effects of sedimenting a meshwork of collagen fibres in the presence of hyaluronic acid*

Concn. of collagen (mg./ml.)	Concn. of hyaluronic acid complex (mg./ml.)	Concn. of hyaluronic acid complex in supernatant (mg./ml.)	Wt. of pellet (mg./mg. of collagen)	Action of hyaluronidase on pellet
2.5	0.6	0.53	51	Yes
2.5	—	—	10	No
—	0.6	0.60	No ppt.	—
Difference (mg.) ...	...	0.07	41	
Difference (% of control) ...	...	-12	+410	

Table 6. *Influence of various collagen concentrations on the weight of material sedimented at constant hyaluronic acid concentration*

The most concentrated solution of collagen which could be prepared was 3.2 mg./ml. and this is taken as 100%. The concentration of the hyaluronic acid complex was 0.5 mg./ml. Pellets made in the absence of hyaluronic acid weighed 12–14 mg./mg. of collagen.

Relative concn. of collagen (at const. concn. of hyaluronic acid complex) (%)	$\frac{\text{(Wt. of pellet made in the presence of hyaluronic acid)}}{\text{(Wt. of control pellet made in the absence of hyaluronic acid)}} \times 100$
80	142
70	123
60	124
40	116
20	107

Table 7. *Influence of various hyaluronic acid concentrations on the weight of material sedimented at constant collagen concentration*

The most concentrated solution of hyaluronic acid complex which could be prepared was 2.9 mg./ml. and this is taken as 100%. The concentration of collagen was 1.6 mg./ml. The pellet made in the absence of hyaluronic acid weighed 13 mg./mg. of collagen.

Relative concn. of hyaluronic acid complex (at const. concn. of collagen) (%)	$\frac{\text{(Wt. of pellet made in the presence of hyaluronic acid)}}{\text{(Wt. of control pellet made in the absence of hyaluronic acid)}} \times 100$
40	141
20	121
10	110
2.5	107
0	100
30	130
+ heated hyaluronidase	
30	100
+ active hyaluronidase	

negligible and its effect on the sedimentation measurement was small (Johnston, 1955) compared with that of the particle-particle interaction of hyaluronic acid. The relative viscosity of umbilical-

cord extracts decreased with increasing rate of shear in the Couette viscometer, as is known for other hyaluronic acids (e.g. Ogston & Stanier, 1950). The variation of relative viscosity with shear rate depended on concentration. The relative viscosity measurements are summarized in Fig. 1, and are compared with results on human and ox synovial fluid (Fessler *et al.* 1954).

Compression of mixtures of fibres, polysaccharide and buffer. A meshwork of collagen fibres is formed when a solution of neutral-salt-soluble collagen is warmed to 37° (Jackson & Fessler, 1955; Gross, Highberger & Schmitt, 1955). This property was used to generate a system of fibres intimately dispersed through a solution of hyaluronic acid. Concentrated solutions of collagen and hyaluronic acid (bovine synovial fluid) were mixed in various proportions and a meshwork of collagen fibres was produced *in situ* by warming to 37°. The resulting gel was compressed in the ultracentrifuge into a pellet. This pellet contained more water than a control in which the collagen-fibre meshwork was centrifuged in the absence of bovine synovial fluid, or in the presence of bovine synovial fluid degraded by hyaluronidase. During centrifuging hyaluronic acid was carried down by the pellet, as the concentration of hyaluronic acid in the supernatant was less than in the original solution. A pellet made in the presence of hyaluronic acid had a firm, elastic consistency which it lost upon subsequent incubation with hyaluronidase, when squeezing caused partial collapse with loss of fluid. Control pellets made from collagen-fibre meshworks in the absence of intact hyaluronic acid were much smaller, compacted masses without resilience, and were not altered by incubation with hyaluronidase. Table 5 shows results under optimum conditions; Table 6 shows the effect of varying the collagen concentration and Table 7 the results of varying the concentration of hyaluronic acid. The starting solutions were the most concentrated ones that could be made and clarified before use; more concentrated solutions were so viscous that prolonged centrifuging was needed for clarification, during which appreciable sedimentation of the wanted material itself occurred. Optimum condi-

tions arose when both components were as concentrated as possible. These conditions could not occur in experiments in which the concentration of one component was varied while that of the other was kept constant, because buffer was added. The use of hyaluronidase showed that only the hyaluronic acid part of synovial fluid contributed to the effect. The difference in weight of pellets obtained in the presence and absence of intact synovial fluid must practically all be due to water held in the pellet; the total solid constituents of the fluids mixed in an experiment could account for only a few per cent of the differences in weight of the pellets.

DISCUSSION

This investigation had two aims: (1) to compare the physicochemical properties of hyaluronic acid extracted by the mildest means from umbilical cords with those of other hyaluronic acids; (2) to see how hyaluronic acid contributes to the structure of Wharton's jelly: this has led to a more general postulate of how polysaccharide may contribute to a mechanical function of connective tissue and this postulate was tested on a model.

Properties of hyaluronic acid extracted from Wharton's jelly

Table 4 and Fig. 1 indicate that hyaluronic acids from human umbilical cord and synovial fluids are similar, and differ from those of sheep and ox synovial fluids, in particular in the dependence of relative viscosity on shear rate. The parameters calculated for the hyaluronic acid particle indicate a hydrated open coil, with some stiffness and a particle weight in the range of 4×10^6 molecular-weight units. The particle weight of these impure preparations of human-umbilical hyaluronic acid, extracted under very mild conditions, is in agreement with the findings of Laurent (1957) and Laurent & Gergely (1955), who used material that had been exposed to more severe conditions during purification. Blumberg & Oster's (1954) value of 8×10^6 is rather higher and Balazs, Varga & Gergely's (1957) value of 2.7×10^6 and 1.44×10^6 is somewhat lower, as are the findings of Christiansen & Jensen (1952) and Jensen & Djurtoft (1954). The model of a randomly kinked coil with some stiffness agrees with Laurent's extensive studies; the value found for the hydrated volume can only be taken to indicate a high hydration.

Structure of Wharton's jelly

From the reported studies, the following structure is deduced. Wharton's jelly is a matrix of fine fibres, probably largely collagen fibres, polysaccharides, proteins, salts and water. A large part of the polysaccharide and most of the soluble protein

is only loosely held; it can be leached out after maceration or centrifuged out from swollen cords. A further part of polysaccharide can be extracted after more drastic mechanical subdivision. The whole matrix has a curious property which must be explained in terms of its structure: it imbibes water and holds it intimately; no anatomical confining structure is discernible and a large part of the water is held after cutting up a moderately swollen cord. This is the same phenomenon as the bleb or 'Ranvier bulla' produced by subdermal injection of fluid, and a Ranvier bulla can also be raised in umbilical cords.

The following explanation is suggested: water enters the jelly because of an osmotic difference between it and the bathing fluid. The osmotic difference is due to protein and in part to the polysaccharide within the cord. The water interacts with the polysaccharide to form a viscous solution which is distributed throughout a meshwork of fibres. A structure with mechanical rigidity results; deformation necessitates flow of the very viscous solution and this in turn is prevented by a tangle of fibres. An alternative view is that the polysaccharide particles are sufficiently entangled with each other and the fibres to prevent appreciable displacement of polysaccharide when moderate pressure is applied to the swollen cord, whereas at higher pressure a solution of polysaccharide is released. The experiments with synthetic mixtures of fibres and hyaluronic acid support these views.

Synthetic mixtures of fibres and hyaluronic acid

The results given in Tables 5-7 show that a mixture of fibres, hyaluronic acid and water can be made which will withstand temporary, yet considerable, compression. The importance of hyaluronic acid and of its being intact was shown by the studies with hyaluronidase. The role of the hyaluronic acid was most probably a mechanical one during centrifuging of the gel, but it may also have influenced, secondarily, the formation of the collagen-fibre meshwork. A given quantity of soluble collagen might polymerize into fibres of different size and different mutual orientations according to the environment, resulting in meshworks of different compressibility. Such an action of hyaluronic acid has not been excluded; it would be necessary to examine a large number of samples from gels in the electron microscope, and further possible changes during preparation of the samples could occur.

The synthetic mixture of fibres and hyaluronic acid had mechanical properties similar to those of Wharton's jelly. Direct comparison would require knowledge of the fibre sizes, the distribution of fibres and the concentration and nature of polysaccharide in both systems. Even after extensive

electron microscopy, it would be difficult to obtain such complete descriptions. Thus as the physical composition of Wharton's jelly was not defined, little was to be gained in using its components for the construction of a model system in preference to the analogous bovine materials. The important point is that a system has been made *in vitro* which will withstand temporary compression.

General considerations

Elementary analysis shows that bone and cartilage cannot be the only structures that are under compressive stress in the body; any small region of the body must be able to withstand some compression. Water occurs throughout the body and is practically incompressible. It would form the basis of a structure withstanding compression, provided that its free flow out of a structure which is being squeezed is hindered. That there is no free-flowing water in the animal body, apart from the secretions and the vascular system, is apparent when one cuts open an animal. Positive resistance to flow of liquid is shown if an intradermal injection is made: a bleb or Ranvier bulla is raised and little free fluid is obtained by cutting this open. Hyaluronidase interferes with the system which hinders the flow of water, as is shown in its facilitation of subdermal infusions. By analogy with the results on Wharton's jelly and synthetic mixtures of fibres and solutions of hyaluronic acid, it is proposed that: (1) a mixture of water, polysaccharide and fibres can form a structure which will withstand temporary compression by hindering the flow of water out of it, and (2) that such structures locally occur in the animal body.

Such a structure involves two principal frictional interactions: (1) between water and polysaccharide, and (2) between the resulting solution and fibres. A measure of the first interaction is the sedimentation coefficient of hyaluronic acid in water (buffer), whereas the second interaction is a function of the viscosity of the polysaccharide solution and the pore size (regarding the meshwork of fibres as a porous plug). For a solution of given viscosity, the second frictional interaction is increased if the effective pore size is decreased by placing the fibres more closely together. At a limiting pore size the two frictional interactions are equal, and at smaller pore sizes a pressure gradient would produce filtration of water out of the system as the frictional interaction between water and polysaccharide is less than between polysaccharide solution and fibres. For hyaluronic acid from bovine synovial fluid and from human umbilical cord, the limiting pore size was calculated approximately as 0.5–1.0 μ , by equating the frictional interactions, defined by the known sedimentation coefficients and relative viscosities, and making the considerable

approximation of taking Poiseuille's Law to hold under these conditions. The order of magnitude of the pore size is reasonable, and indeed Ogston & Stanier (1950) had found that hyaluronic acid is filtered from bovine synovial fluid by sintered-glass filters of 1 μ pore size. Hyaluronic acid from human synovial fluid and extracts of human umbilical cord could just be retained on such filters by using a very slow flow rate. Although this method is impracticable for preparative purposes, it was clear that pores of that order of magnitude would be adequate in the proposed fibre meshwork.

The approximate pore size in the synthetic mixture of fibres and hyaluronic acid can be calculated. The total volume of fibres/ml. is known from the concentration and density of collagen. If a mean fibre diameter is assumed, the total fibre length/ml. follows. One may now either (i) simply allot all the water space as a uniform cylindrical envelope to the fibres and take the mean distance between fibres as twice the thickness of the cylindrical envelope, or, better, (ii) apply the statistical treatment of Ogston (1958), with the approximations for a network composed of very long straight fibres. Collagen fibres made by warming a solution of soluble collagen to 37° have diameters principally within the range 100–1000 Å (Fessler, 1956). If one assumes a mean diameter of 400 Å, a pore space of 0.85 μ results for the experiment of Table 5 by the simple calculation (i); by Ogston's treatment (ii) a mean tangential separation ($2\bar{D}$) of 0.8 μ , and a modal value ($2D_m$) of 0.65 μ . These values vary directly with the assumed fibre diameter (e.g. by simple treatment, 0.65 μ for 300 Å fibre diameter, 1.1 μ for 500 Å fibre diameter). Thus the pore size of meshworks which resist compression is calculated as being of the same magnitude as that calculated, and experimentally known, to be necessary for hyaluronic acid to be held back.

How far can a random dispersion of fibres be considered as a meshwork for these purposes? The difficulty of teasing apart fibres, formed by warming a solution of soluble collagen, indicates that they are intertangled. The degree to which a random meshwork of fibres may be regarded as intertangled will increase as the length of the fibres increases relative to their width and their distance apart. Fibres 20–100 μ long, and longer, were readily found in Wharton's jelly; their width was of the order of 0.05–0.1 μ , their distance apart of the order of 1 μ (by calculation of 'pore size'). This makes it unlikely that appreciable movement of a part of a fibre could occur without its coming up against another fibre, i.e. its becoming intertangled. For comparison, the length to width ratio of these collagen fibres is of the order of that of 1 m. long spaghetti, but the fibres are probably a good deal stiffer than those of cooked spaghetti (judged by

electron-microscopic appearance) and are calculated to be some 10 diameters apart. It is reasonable that in the absence of a viscous filler such a structure should be collapsible, as indeed is a meshwork of collagen fibres generated in buffer. The maintenance of the dispersed meshwork is thus seen to be dependent on the viscosity of the fluid filling the meshwork, as is found experimentally, and the meshwork in turn traps the viscous fluid. For purposes of holding back viscous fluid, an appreciable local variation in pore size, which is likely to occur, will be less important than any co-operative phenomenon in which a channel would be opened; the more the fibres are intertangled, the less likely is it that there is a channel, or that a channel would be formed as a result of a co-operative set of displacements.

Instead of considering the system as a meshwork filled with viscous fluid, one may also treat it as a viscous fluid into which an increasing number of long particles are placed. These would greatly increase the viscosity even in the absence of particle-particle interaction (Simha, 1940) and a visco-elastic structure is likely to result if the interaction is of the order of entanglement. Indeed, hyaluronic acid solutions alone show visco-elastic (recoil) behaviour at the concentrations here used.

The following regions of animal bodies contain hyaluronic acid (Meyer, 1947; Arteta, 1956; Boas, 1949), are relatively poor in cells and are exposed to local compression: embryological structures in which rapid alterations of cells have to occur, umbilical cord, placenta, vitreous humour, areolar tissue (as in skin, sex skin of Rhesus monkey, cock's comb and some near environs of nerves and blood vessels) and joint fluid. Fibres, often quite fine ones, are present in these regions [e.g. vitrosin fibres of vitreous humour (Pirie, 1949; Gross, Maltoltsy & Cohen, 1955)], with the exception of joint fluid, which is a mobile lubricant. The suggested compound structures of hyaluronic acid, fibres and water may thus play a physiological role in these locations. The place of hyaluronic acid could be taken by any other macromolecule, of the open-coil type of structure, offering a high resistance both to fluids' being moved through it and to its being moved with fluid through a meshwork or set of narrow channels. Thus chondroitin sulphate in cartilage may play a similar role, though no doubt the electrical interactions due to the large number of charged groups are also important here.

The elegant experiments of Day (1950) suggest that some type of sieve is necessary to hold back hyaluronic acid particles in fascia. No systematic structure of this kind could be found in Wharton's jelly, yet the hyaluronic acid was held in the swollen cord. The ready extraction of hyaluronic acid from the jelly, by water or buffer, makes it

unlikely that a chemical binding is important. The experiments with collagen fibres and bovine synovial fluid indicate that hyaluronic acid can be held in a random arrangement of fibres, and that the hyaluronic acid is concentrated on compression of the meshwork. Although frictional interactions have here been shown to suffice, other interactions may also be important *in vivo*.

SUMMARY

1. A structure is proposed for non-skeletal connective tissue exposed to limited compression; its essential components are water, mucopolysaccharide and fibres. The proposal is based on studies of umbilical cord and of mixtures of collagen fibres and hyaluronic acid.

2. Wharton's jelly of human umbilical cord was investigated with the electron microscope and by swelling studies.

3. Extracts of such Wharton's jelly were made by mild methods. Measurements in the ultracentrifuge and Couette viscometer showed the hyaluronic acid in the extracts to be similar to human synovial fluid hyaluronic acid. The hyaluronic acid particle is approximately described as an open, partially stiff coil, of particle weight 4×10^6 molecular-weight units and having a large hydrodynamic hydration.

4. Intimate mixtures of collagen fibres and hyaluronic acid were ultracentrifuged. The compound system resisted limited compression; hyaluronic acid and water were trapped in the compressed meshwork.

5. The frictional interactions in such systems are discussed.

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Mode of Action of Testicular Hyaluronidase

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Hyaluronidase facilitates the movement of aqueous solutions along a pressure gradient through connective tissue (Duran-Reynals, 1929). Although chemical studies have shown the enzyme to act on certain polysaccharides found in connective tissue (Meyer & Rapport, 1952), it is not clear how the movement of water is thereby facilitated. *In vitro*, hyaluronidase causes a marked reduction in the viscosity of hyaluronic acid solutions. When water is moved through connective tissue along a pressure gradient, at least two frictional interactions are likely to occur: (1) between water and mucopolysaccharide of the tissue, and (2) between mucopolysaccharide and the confining structures of the tissue. Hyaluronidase could facilitate the movement of fluid by altering either or both of these interactions and the following experiment was devised to elucidate this problem. A measure of the first interaction, between water and mucopolysaccharide, can be obtained *in vitro* by observing the movement of mucopolysaccharide through water (buffer) in the analytical ultracentrifuge. A measure of the second interaction, between mucopolysaccharide solution and confining structures, is obtained *in vitro* from the relative viscosity of such solutions. The mucopolysaccharide for these experiments was prepared from bovine synovial fluid by Ogston & Stanier's (1950) method of ultrafiltration; the material had been defined by them and called hyaluronic acid complex or ultrafiltrate residue (U.F.R.). It was degraded stepwise with hyaluronidase and the sedimentation and viscosity properties of the products were measured at

different concentrations. It was concluded that the initial action of hyaluronidase reduces the interaction of mucopolysaccharide solution with the confining structures, and that the interaction of water with mucopolysaccharide is affected only when considerable degradation has occurred. This suggests that when hyaluronidase is used as a spreading agent it causes hyaluronic acid to be moved through the tissues with the injected fluid, rather than facilitating the movement of fluid through the hyaluronic acid. The action of trypsin on the hyaluronic acid complex of bovine synovial fluid was also studied with the ultracentrifuge.

EXPERIMENTAL

Synovial fluid. This was collected and stored as described by Fessler, Ogston & Stanier (1954) from cattle at the slaughterhouse of the Oxford Co-operative Society. Merthiolate [sodium ethyl mercurithiosalicylate solution no. 45 (0.1%, w/v), monoethanolamine (0.1%, w/v) Boots Pure Drug Co. Ltd., Nottingham] was added to a final concentration 1:34 000 (w/v).

Buffer. This was 0.2 M-NaCl-7.7 mM- Na_2HPO_4 -2.3 mM- KH_2PO_4 , pH 7.3.

Enzymes. Hyalase was obtained from Benger Ltd. and Trypsin (reagent grade) from British Drug Houses Ltd.

Preparation of hyaluronic acid complex by ultrafiltration. This was carried out by the method of Ogston & Stanier (1950). Dilute hyaluronic acid complex (U.F.R.) solution was clarified by ultracentrifuging (Spinco rotor no. 40, 39 000 rev./min., 40 min.), concentrated by further ultrafiltration and the viscous remnant dialysed against standard buffer. A marble was enclosed in the dialysis bag for stirring.