CHANGES IN THE PHYSICAL CHARACTERISTICS OF THE HYALURONATE OF GROUND SUBSTANCE WITH ALTERATIONS IN SODIUM CHLORIDE CONCENTRATION[1]

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Hyaluronic acid was first isolated in 1934 by Meyer and Palmer (1) from the vitreous humor of the cattle eye. It has since been isolated from the ground substance of some connective tissues, synovial fluid, cartilage, cornea, umbilical cord, bacteria, and possibly identified in the ground substance of other material (2, 3). The substance is a high polymer with a monomer of a disaccharide, β-glucuronido-1-3 N-acetylglucosamine, constituting the repeating unit (4). Each of these monomers contains an ionizable carboxyl group. The compound is one of a large group of natural polyelectrolytes.

The connective tissue, in the ground substance of which hyaluronic acid is found, is a ubiquitous material, and thought to be of importance in support, ion transport, storage, and repair (3). Changes in the physical characteristics of hyaluronic acid may be of importance in the exercise of these functions.

Studies of the physical properties of hyaluronic acid by various methods have been reported (5–14). We have studied the relative changes in certain biophysical measurements of hyaluronate solutions including flow birefringence, viscosity, sedimentation and light scattering as a function of sodium chloride concentration to determine the role of the sodium ion in such physical changes.

Methods and results. Studies of flow birefringence, viscosity, sedimentation and light scattering were made on solutions of sodium hyaluronate at various concentrations of NaCl maintained at pH 5.0 with acetate buffer. This value was selected in order that the studies be performed at a pH optimal for the action of hyaluronidase, in anticipation of further enzymatic studies. From electrometric titrations, the material is estimated to be about 80 per cent ionized at this pH (1, 15). Hyaluronate from two sources was studied: human umbilical cord and streptococcal capsule. They were prepared by an alcohol precipitation method (16). Proteolytic digestion was used in the umbilical cord specimen. Chemical analyses of the two materials are shown in Table I, and show that the preparations are essentially protein-free.

A complete series of measurements were made using the umbilical cord specimen and confirmatory determinations for the streptococcal hyaluronate were also performed. The relative changes in the measurements were the same in both specimens and, for the sake of brevity, the values for umbilical hyaluronate only will be shown.

Flow birefringence. Flow birefringence on solutions at 0.25 per cent concentration of hyaluro-

TABLE I

Chemical analysis of hyaluronate specimens

	Nitrogen	Hexosamine	Sulfate	Hexos./ nitrogen	Uronic acid (Dische)	Uronic acid/ nitrogen
Human umbilical cord MV 62	3.03%	38.0%	<0.4	0.98	45.5%	1.08
Strep. capsule MV 69A	3.06%	40.9%	—	1.05	44.0%	1.04

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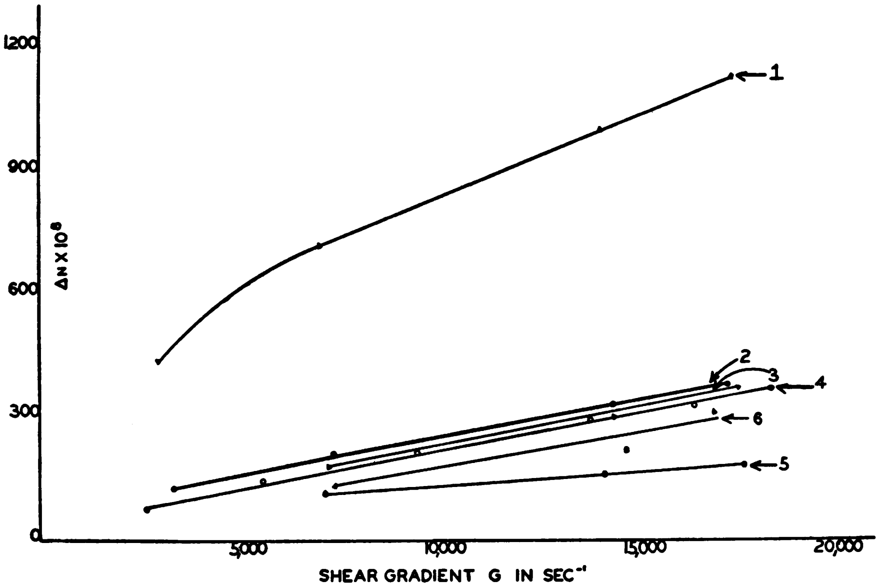

FIG. 1. FLOW BIREFRINGENCE, Δ_n

Human umbilical cord hyaluronate concentration 0.25 per cent.

Flow birefringence. Δ_n as a function of shear gradient for solutions at various sodium chloride concentrations at pH = 5.0 (acetate buffer).

Sodium ion strength; curve 1, 0.005; curve 2, 0.085; curve 3, 0.115; curve 4, 0.25; curve 5, 1.6; curve 6, 2.25.

nate was determined at room temperature on the small flow birefringence instrument described by Edsall, Rich, and Goldstein (17). Measurements of angular velocity were made with a Strobotac lamp. Shear gradients up to 19,000 sec.$^{-1}$ were obtained. Extrapolations to zero shear gradient and to 18,000 sec.$^{-1}$ were made for purposes of comparing the different solutions. Figures 1 and

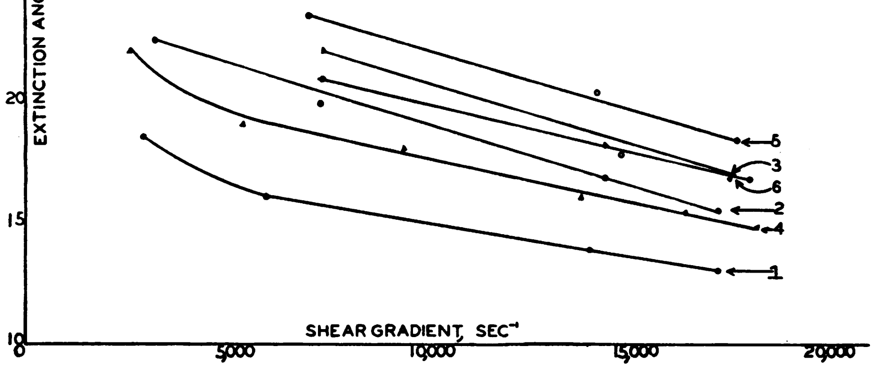

FIG. 2. FLOW BIREFRINGENCE, EXTINCTION ANGLE

Human umbilical cord hyaluronate concentration 0.25 per cent.

Flow birefringence extinction angle as a function of shear gradient for solutions at various sodium chloride concentrations at pH 5.0 (acetate buffer).

Numbering of curves as in Figure 1.

2 show Δ_n and extinction angle *vs.* shear gradients (where these quantities have the usual meanings [18]) for each of the added salt concentrations. Light of wavelength 517 mμ was used and the path length was 3.87 cm.

In all cases, measurements at shear gradient of approximately 18,000 sec.$^{-1}$ were easily made although they became increasingly more difficult as the salt concentration was increased and the shear gradient decreased. Accuracy of extrapolation to zero shear gradient was therefore limited. The value for flow birefringence Δ_n and angle of extinction at shear gradients of 18,000 sec.$^{-1}$ are shown as a function of sodium ion strength in Figure 3. The flow birefringence Δ_n decreases, and the extinction angle increases with an increase in the salt concentration in the solution (Figures 1, 2, 3). There is a marked change in these values below sodium ion strength of approximately 1.3 and only small changes with addition of salt beyond this concentration.

Viscosity. Viscosity was measured in an Ubbelöhde (19) viscometer with a capacity of 3.75 cc. and a distilled water flow time of 126 seconds. The apparatus was maintained at 37° C. ± 0.05 in a constant temperature bath. Relative, specific and intrinsic viscosities were determined for each of the solutions of different sodium ion strength. Intrinsic viscosities were determined by extrapolating the specific viscosity η sp (η sp = η r − 1, where ηr is the viscosity of the solution relative to that of the solvent) divided by concentration, to zero concentration (Figure 4). In the case of curve 1, to determine the intercept, a plot of c/η spec. *vs.* $\sqrt{c}$ was made and the resultant straight line extrapolated to zero concentration (20). Intrinsic viscosity as a function of the sodium ion strength is shown in Figure 5.

It has been noted by previous investigators (5–8, 21, 22) that increasing the ionic strength of the salt decreases the viscosity of the hyaluronate solution. This was also found to be the case for our material. The major changes in the viscosity occur with the addition of small amounts of salt. After approximately 0.5 M, the drop is gradual and after 1.3 M, there is very little change with the addition of even large amounts of salt.

Balazs and Laurent (8) have shown by measurements of intrinsic viscosity as a function of concentration of hyaluronate that the material be-

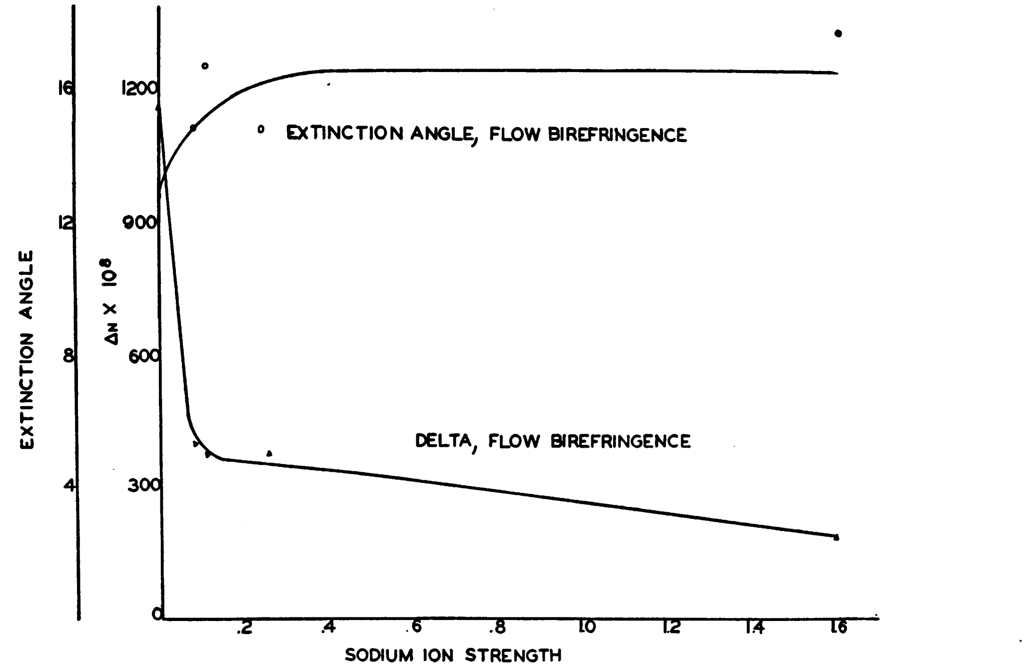

Fig. 3. Flow Birefringence as a Function of Sodium Ion Strength

Human unbilical cord hyaluronate concentration 0.25 per cent. pH = 5.0 (acetate buffer).

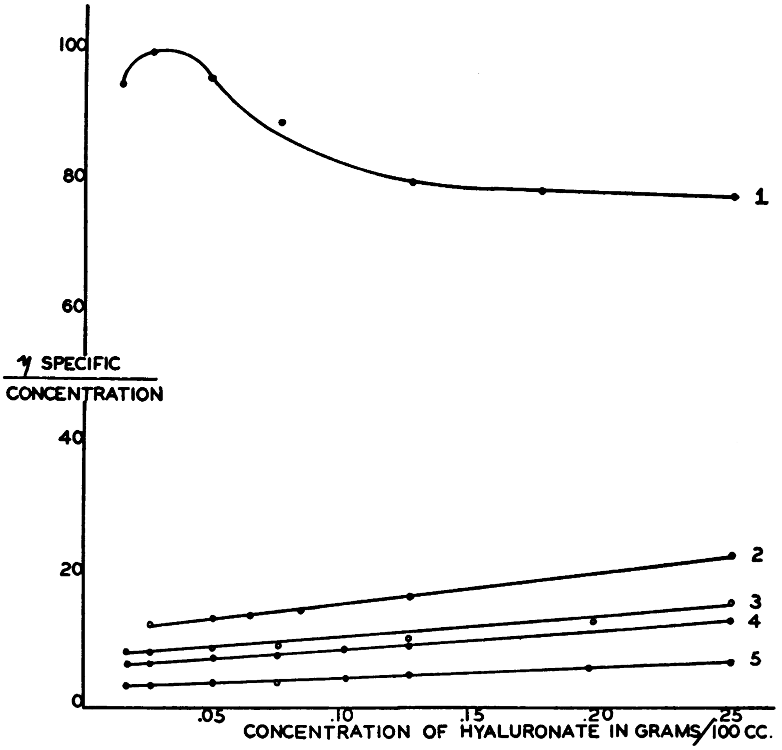

Fig. 4. Viscosity Studies

Human umbilical cord hyaluronate.

η spec./conc. as a function of concentration of hyaluronate, for solutions at various sodium chloride concentrations at pH = 5.0 (acetate buffer).

Sodium ion strength; curve 1, .005; curve 2, .025; curve 3, .085; curve 4, .115; curve 5, 1.6.

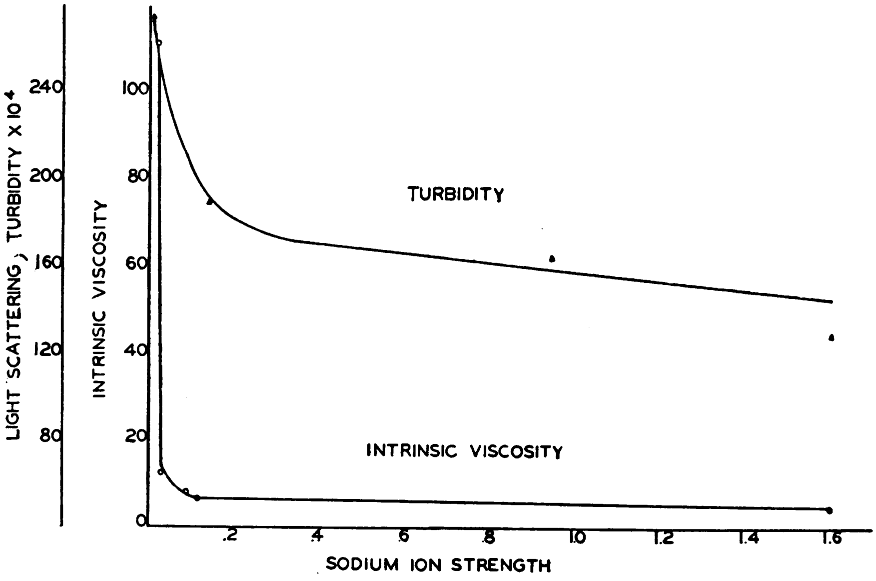

Fig. 5. Human Umbilical Cord Hyaluronate—Intrinsic Viscosity, and Turbidity (from Light Scattering Studies) as a Function of Sodium Ion Strength

haves as a polyelectrolyte. In distilled water, the curve of η sp/c *vs.* c shows an impressive rise as the concentration approaches zero. Our studies confirmed their results (curve 1 of Figure 4). They achieved a very low sodium ion concentration by dialysis and electrodialysis. The solutions made in distilled water in the present studies were undialyzed and contained 5 milliequivalents of sodium ion per 100 cc. as determined by flame photometry. Hence, the rise in the curve was not as striking as it was for Balazs' material which contained essentially no sodium ion.

Sedimentation. Sedimentation constants were determined on the Spinco model E ultracentrifuge. Measurements of the peaks on the photographic plates were made with a microcomparator and by projection. Sedimentation constants were determined by standard formulae (see, for example, reference 23), using measurements on five exposures, each 32 minutes apart, making corrections for temperature and viscosity. Rotor speed was 59,780 revolutions per minute.

Runs at several dilutions of hyaluronate at various salt concentrations were performed and sedimentation at infinite dilution determined by extrapolating 1/S *vs.* c by the method of Ogston (24), where S is the sedimentation constant in Svedbergs and C is the concentration of hyaluronate in grams per 100 cc. Since the sedimentation is difficult to determine at very low concentrations of hyaluronate, there is necessarily considerable error in the extrapolated values.

Values of sedimentation constant at hyaluronate concentration of 0.25 per cent and at hyaluronate extrapolated to zero concentration are shown in Table II. The extrapolated value of sedimentation appears to be greatest at low salt concentrations and decreases with the addition of salt. It is of interest that the contrary trend is noted for hyaluronate concentration of 0.25 per cent and consideration of these values uncorrected for concentration might lead to an erroneous impression.

Hyaluronate from both umbilical cord and the streptococcal capsule gave extremely sharp peaks (Figure 6). With decrease in concentration, these were found to broaden somewhat. It is suggested that this hypersharp peak may not be due to low polydispersity but rather to interaction between the particles.

Light scattering. Light scattering studies were performed on the Aminco light scattering microphotometer (25), using the green line of mercury, $\lambda = 546$ millimicrons. The absolute turbidity τ (a measure of the amount of light scattered by the solution [26]) is measured by comparing the intensity of the scattering at an angle of 90 degrees with that of Ludox, an aqueous suspension of silica (25, 27). Angular measurements were made at 45°, 90°, and 135°. Solutions of hyaluronate were cleared of dust and particulate matter by centrifugation and filtering, using suitable fritted glass filters.

Starting with the buffer alone containing salt at a specific concentration, measured amounts of hyaluronate dissolved in the same buffer and at the same salt concentration were added serially. Turbidity and angular measurements were made at each concentration of hyaluronate. This gives a family of light scattering measurements at different concentrations of hyaluronate for different salt concentrations. Hc/τ (where the optical factor $H = 3.54 \times 10^{-6}$ [9] and c is in gm. per cc.) was found to be essentially independent of concentration for each of these solutions in a range from 3×10^{-4} to 9×10^{-4} gm. per cc. of hyaluronate. From these curves a value for turbidity corrected for dissymmetry at hyaluronate concentration of 5×10^{-4} gm. per cc. was determined and is shown as a function of sodium ion strength in Table III and Figure 5. The corrected turbidity *versus* ionic strength of sodium was rechecked by measurements on hyaluronate solutions at a concentration of 0.25 per cent in buffered solutions of different salt concentrations. The turbidity de-

TABLE II

Sedimentation studies *

Preparation	Sodium ionic strength	Sedimentation at hyaluronate concentration 0.25%	Sedimentation for concentration of hyaluronate extrapolated to zero
Umbilical cord	0.025	0.76	6.3
	0.085	0.89	4.2
	0.175	1.39	
	0.250	1.54	2.8
	1.600	1.52	

* Human umbilical cord hyaluronate. Sedimentation (in Svedbergs) at hyaluronate concentration of 0.25 per cent, and for hyaluronate concentration extrapolated to zero, in solutions containing sodium chloride at various concentrations (pH 5.0 acetate buffer).

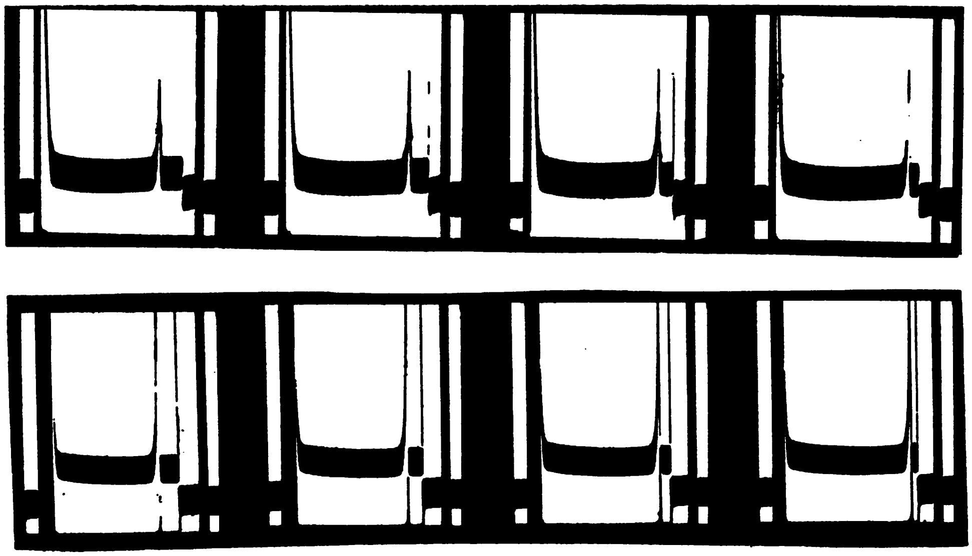

FIG. 6. ULTRACENTRIFUGE STUDIES

Rotor speed 59,780 rpm. Exposure interval, 32 minutes. Concentration of hyaluronate 0.5 per cent. Solutions are made up in 0.1 M acetate buffer (pH = 5.0) and contain 0.15 M sodium chloride. (a) Human umbilical cord hyaluronate. (b) Streptococcal capsule hyaluronate.

creases with increase in sodium chloride concentration. The major decrease occurs below a sodium ion strength of 1.5 and changes only slightly with changes in salt concentration beyond this value. The dissymmetry measured at 45° decreases slightly with the salt concentration. See Table III.

DISCUSSION.

There are profound changes in the physical properties of hyaluronate with changes in salt concentration. The major changes occur in a sodium ion concentration range just below that of physiological saline where there is an exquisite sensitivity to this ion. Beyond a sodium ion strength of approximately 0.15, the hyaluronate is relatively insensitive. It is of interest that in preliminary studies, similar but more profound changes have been found with chondroitin sulfuric acid (28, 29).

There have been many studies of the physical characteristics of polyelectrolyte solutions, *i.e.*, high polymer molecules having ionizable groups as part of the repeating unit (30). Studies on the chemical structure of hyaluronic acid have shown it to fit into this classification (4). The present data on its behavior in salt solutions are, in general, what one would expect of such a charged polymer.

It should be understood that interpretation of such data in terms of physical models can be only approximate and general. Some remarks, however, can be made about the probable changes in the hyaluronate particle and its solutions.

The increase in sedimentation constant and in turbidity as the ionic strength is decreased below 0.15 molar suggests that the particles aggregate in this low salt concentration environment. The increased viscosity and flow birefringence at low salt concentration further strengthens this hypothesis, although consideration of these values alone

TABLE III

Light scattering studies *

Sodium ion strength	τ 90 corrected	Dissymmetry at 45°
0.005	28×10^{-3}	4.84
0.145	19×10^{-3}	4.43
0.915	17×10^{-3}	4.35
1.6	13×10^{-3}	4.38

* Human umbilical cord hyaluronate. Turbidity at 90° corrected for dissymmetry, and dissymmetry at 45°, for hyaluronate concentration of 5×10^{-4} gram per cc. in solutions containing sodium chloride at various concentrations (pH 5.0 acetate buffer).

would be compatible with other explanations, *e.g.*, uncoiling of the polymer chain.

Regardless of the interpretation of the extrapolated values of the data, it is clear that solutions of hyaluronate undergo marked physical changes when the salt concentration is changed. Decreasing the salt concentration tends to make the solution more "rigid" and "stiff." Increasing the salt concentration has the reverse effect. Hence, the physical properties of this constituent of some of the ground substance, and salt and water variations appear to be intimately related.

Our studies were performed *in vitro* under conditions differing from physiological as to concentration, pH, the presence of other materials, etc. It remains to be shown that hyaluronate reacts in a similar manner *in vivo* and is important as a factor in the control of salt and water in the body as in edema, inflammation, dehydration and other states in which water may be shifted between body compartments.

SUMMARY

1. Biophysical studies on two specimens of isolated hyaluronate in solutions containing different concentrations of sodium chloride have been performed. The studies include flow birefringence, viscosity, sedimentation and light scattering.

2. There are marked changes in these measurements with change in sodium ion strength. The values (extrapolated to infinite dilution of hyaluronate) suggest that the particles aggregate at low salt concentrations and disaggregate with the addition of salt. These changes occur in a region of physiological interest.

3. There is an intimate relationship between the physical properties of hyaluronate solutions and sodium chloride concentration. If this material acts in a similar manner *in vivo,* it may be of importance as a factor in the control of salt and water metabolism as in edema, inflammation, dehydration and other states in which water may be shifted between body compartments.

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