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Hadidian, Z. and Pirie, N. W. 1948. The preparation and some properties of hyaluronic acid from human umbilical cord. *Biochemical Journal.* 42 (2), pp. 260-265.

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activity shown. From evidence so far available it is not, however, possible to formulate the factors to which the lowered potency of the glucuronides is due.

SUMMARY

The isolation and purification of the monoglucuronides of stilboestrol, hexoestrol and dienoestrol, and their chemical and oestrogenic properties, are described.

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The Preparation and Some Properties of Hyaluronic Acid from Human Umbilical Cord

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Hyaluronic acid has now been known for ten years and in this period the literature about it has become both extensive and inconsistent. A number of substances with varying properties has been isolated by different methods from umbilical cord, skin, vitreous humour, synovial fluid, tumours and haemolytic streptococci. In spite of the differences in their properties, all are called hyaluronic acid if they give more or less viscous solutions and if they are composed for the most part of glucuronic acid and acetylglucosamine. There is no clear evidence whether several substances exist with somewhat similar properties, or whether the observed variations are due to contamination with unrelated material, or to partial destruction of originally homogeneous hyaluronic acid during the isolation.

As a prelude to an investigation of the effect of various agents on the enzymic decomposition of hyaluronic acid we have tried to define the conditions under which a product with more clearly defined properties can be prepared. This was necessary because crude preparations differed greatly in the rates at which they lost viscosity with testis hyaluronidase. Contamination of the preparation by substances activating or inhibiting the enzyme is the most obvious explanation of this phenomenon.

The elementary composition of a hyaluronic acid preparation is of little value as a criterion of purity.

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The likely contaminants have carbon and hydrogen contents similar to those expected for hyaluronic acid. The nitrogen content is a good index of heavy contamination with protein, but no great confidence should be placed on a 'correct' nitrogen value, for this is easily attained by the simultaneous presence of comparable amounts of protein and a nitrogen-free polysaccharide. There is substantial agreement that hyaluronic acid contains neither phosphorus nor sulphur but freedom from sulphur has not often been achieved in making preparations of hyaluronic acid.

Apart from proximate analysis, hyaluronic acid can be examined for its constituents: glucosamine, glucuronic acid and acetic acid; and the equivalent weight can be determined by titration with alkali. These measurements have not often been made on the preparations used as substrate in studies on hyaluronidase. Where they have been made considerable variation is apparent, especially in the case of determinations of the equivalent weight. This value should be 379 for the free acid, on the assumption that the molecule is so large that the ends may be neglected, but Meyer, Smyth & Dawson (1939) and Meyer & Chaffee (1940) have given a series of values ranging from 400 to 640, and our own values are within this range. It seems likely that the carboxyl group in at least part of the glucuronic acid is not free. The existence of this type of linkage robs the equivalent weight of some of its value as a criterion of purity.

Viscosity has been widely used as an index of purity and of the extent to which degradation of the hyaluronic acid has been avoided during the preparation. This is legitimate if we assume that the native acid, in all the various sites where it occurs, has the same original viscosity, for many treatments are known which reduce the viscosity but none that increase it.

In Table 1 published values for the viscosity of preparations of hyaluronic acid are collected. It is clear that there is no uniformity in these values, and it is interesting to note that some of these preparations have been described by their producers as undegraded although the viscosity comes low in the list. For comparison the values for fractions made by the methods to be described in this paper are included in the same table.

acid but that the curve steepens as the concentration increases. These two factors have been disregarded in compiling the last column in Table 1 so that an approximate comparison of the various preparations can be made. In each case a value for the relative viscosity* at the standard concentration of 1 g./l. has been derived from the relation

$$1 + \frac{\text{relative viscosity} - 1}{\text{conc. at which viscosity was measured}}.$$

This approximation tends to exaggerate the viscosities of those products that were measured at the higher concentrations, but no better method of getting comparative figures seems to be available. With most of these preparations the curve relating relative viscosity to concentration has not been given,

Table 1. *Comparison of the viscosities of various hyaluronic acid preparations*

Source	Ionic environment	Temp. (°)	Concentration (g./l.)	Relative viscosity	Relative viscosity reduced to 1 g./l.	Reference
Cords	0·15 M-NaCl	20	2·5	9·3	4·3	Meyer, Smyth & Dawson (1939)
Cords	0·067 M-Phosphate buffer, pH 7	20	1·0	4·3	4·3	Blix & Snellman (1945)
Streptococci	0·15 M-NaCl	21	1·0	3·8	3·8	Seastone (1939)
Cords	0·1 M-Citrate-phosphate buffer, pH 7	30	3·8	10·0	3·4	Hale (1944)
Synovial fluid	0·067 M-Phosphate buffer, pH 7	20	1·0	2·55	2·55	Blix & Snellman (1945)
Cords	0·66 M-NaCl	34	1·0	2·0	2·0	McClean & Hale (1941)
Vitreous humour	0·15 M-NaCl	21	1·0	1·9	1·9	Seastone (1939)
Vitreous humour	0·067 M-Phosphate buffer	20	1·0	1·85	1·85	Blix & Snellman (1945)
Cords	Borate, chloride, phosphate, acetate mixture; 0·34 M in all, pH 7	25	2·3	2·0	1·4	Haas (1946)
Pleural exudate	0·15 M-NaCl, neutral	20	1·8	1·54	1·3	Meyer & Chaffee (1940)
Vitreous humour	0·5 M-NaCl	25	2·5	1·4	1·16	Madinaveitia & Quibell (1940)
Cords	0·083 M-NaCl, 0·083 M-citrate buffer, pH 4·5	25	16·5	2·6	1·1	Madinaveitia & Stacey (1944)
Cords	0·05 M-NaCl, 0·05 M-phosphate buffer, pH 7	25	1·0	8·2	8·2	This paper

The viscosities given in Table 1 were measured at different temperatures, at different concentrations, and in different ionic environments; part of the variation is due to these factors. At low salt concentrations the viscosity is high and it is greatly affected by slight variation in the salt concentration, but in the range in which most of the values fall the effect of changes in salt concentration is small. It is well known that the increment in the viscosity of a salt solution due to the presence of hyaluronic acid is not proportional to the concentration of hyaluronic

and Blix & Snellman (1945) have found that its slope varies from preparation to preparation.

The technique of fractionation used in this work was tried in order to test the basic idea that 'hyal-

* The terminology used in recent papers has not always been consistent. Here relative viscosity means the ratio of flow time of the test solution to the flow time of a solution with the same salt concentration. Relative viscosity − 1 is a measure of the increase in viscosity due to the hyaluronic acid. It is sometimes called the specific viscosity, but we prefer to call it the viscosity increment.

uronic acid' is a name for a family of substances differing slightly in architecture and greatly in particle size. It would seem that the other workers in the field have tried to remove as much protein as possible and have then precipitated all the more or less viscous material in one fraction in spite of its apparent heterogeneity. In this work the appearance of viscous material in several fractions has not been looked on as a disadvantage; the fractions differ in their properties so much that nothing would be gained by mixing them. There is no satisfactory method of separating a mixture of protein and hyaluronic acid in one operation when the two are present in similar amounts. Digestion with commercial trypsin is not permissible if one intends to use the hyaluronic acid for studies on hyaluronidase because the protease preparations we have used inactivate hyaluronidase; there can be no certainty that separation of the inactivator from the hyaluronic acid has been complete. Several of the preparations referred to in Table 1 were made by removing the protein by shaking with chloroform and amyl or butyl alcohol. This process is very satisfactory for the removal of small amounts of protein in the final stages of a preparation, but the operations are tedious if used at the beginning. We prefer to precipitate most of the protein along with only a small part of the hyaluronic acid as a mucin clot and then to isolate the main part of the hyaluronic acid by the methods to be described.

EXPERIMENTAL

Preparation of hyaluronic acid

Human umbilical cords have been used as starting material in all our work. They are washed carefully and then preserved in acetone. After lying for 1–6 weeks in acetone they are cut up into pieces not more than 1 cm. long and extracted for a few more days with fresh acetone. The acetone is then drained off as completely as possible and the cords are extracted with successive lots of water, using about four times the cords' wet weight of water for each extraction and leaving each one for at least 2 hr. The first two extracts contain little but acetone and may be thrown away, but the later extracts bring out some hyaluronic acid accompanied by much protein. These extracts therefore give a mucin clot on acidification; they are combined and extraction is continued until the amount of clot is small. Five to eight extractions may be necessary. The combined extracts are taken to pH 3 and the clot collected so that it can be worked up for hyaluronic acid along with other fractions grossly contaminated with protein.

The residue is passed through a power-driven meat grinder with $\frac{1}{8}$ in. holes in the plate. To reduce the viscosity the ground material is suspended in 3 vol. of approx. 0·1 M-NaCl, and after some hours it is poured into a cloth and the fluid is expressed by hand. The extraction is repeated twice, and the new NaCl solution is allowed each time to lie in contact with the cord for at least 12 hr. If this work is not carried out in a cold room the fluids should

be kept saturated with chloroform at all times. For each litre of the combined extracts 20 ml. of approx. 5 N-HCl are added with thorough stirring. If the initial washing of the cords was adequate there will be only a small precipitate; this is stirred gently and intermittently during some hours, until it coalesces into a string which is then removed and added to the mucin clot fraction.

Hyaluronic acid can be precipitated from this fluid in several different ways. The conventional methods of precipitation with several volumes of ethanol or acetone are inconvenient because of the bulk of the fluid involved. 100 g. dry weight of washed cords (about 40 cords) give 6 l. of extract at this stage. Two alternative precipitation procedures are described.

(a) $(NH_4)_2SO_4$ (300 g.) are added to each litre of the clear acid fluid. Purified hyaluronic acid is not precipitated by $(NH_4)_2SO_4$, but the residual protein in the solution precipitates and brings out some hyaluronic acid with it. The precipitate that separates entangles air forced out of the solution by the salt; this is convenient for it is easier to allow the mixture to stand till all the solid has collected at the top, then to siphon off the subnatant fluid and compact the solid scum by centrifuging, than to de-aerate and centrifuge down the precipitate. To each litre of fluid 50 ml. of pyridine are added and the mixture is shaken vigorously by hand; some of the pyridine remains undissolved in the $(NH_4)_2SO_4$ solution and a little solid separates at the interface. The mixture is allowed to settle, the clear fluid is siphoned off and the interfacial material is compacted by centrifuging. This centrifuging should be done in a covered tube so that the pyridine does not evaporate, because the pellicle remains insoluble only if some pyridine phase is present. The pellicle generally contains little hyaluronic acid. A further 250 g. of $(NH_4)_2SO_4$ are now added. This forces more pyridine out of solution and it brings with it hyaluronic acid which stays at the interface between the two liquids. On centrifuging in closed vessels, it becomes a compact coherent sheet that can be lifted out of the centrifuge tube with a pair of forceps. Approximately equal amounts of hyaluronic acid are present in the original $(NH_4)_2SO_4$ precipitate and in the second pyridine pellicle, but the former may contain as much as 20 % of protein and the latter only 5 % judging from the nitrogen content. In each case the protein can be removed by shaking with chloroform and amyl alcohol according to the Sevag technique.

(b) A comparable method for separating hyaluronic acid from the acid cord extract involves precipitation with acid ethanol in the presence of $(NH_4)_2SO_4$. It offers no advantages over the method already described and involves handling larger volumes of fluid, but there may be other sources of hyaluronic acid with which it might have advantages. Ethanol (1·33 vol.) is added to the acid fluid; a fine gelatinous precipitate separates and this is entangled with air bubbles. The whole precipitate may be sufficiently coherent to float up in 1 hr., thus enabling one to siphon away most of the fluid. If it fails to do this, the air is removed from the precipitate by exposure to vacuum for a few minutes. The precipitate will now sink and can be centrifuged down. This precipitate is suspended evenly in water and reprecipitated by addition of 1·33 vol. of ethanol; on dialysis it goes into solution slowly as the ethanol is removed. Solid $(NH_4)_2SO_4$ is added to the ethanolic supernatant fluid with vigorous mechanical stirring. It dissolves

slowly and about 200 g. are needed to saturate 1 l. On standing, the system separates into two layers; the lower contains much $(NH_4)_2SO_4$ and little ethanol and the upper little $(NH_4)_2SO_4$ and much ethanol. As a rule a precipitate which is relatively uncontaminated hyaluronic acid collects on the interface. When separation has proceeded so far that both liquid layers are clear, as much of each as possible is siphoned off and the interfacial material collected by packing it into a coherent felt by centrifuging. It packs very tightly when centrifuged and should be diluted with water before dialysis, for otherwise a rigid jelly will be formed that dialyzes slowly. The proportion in which these two fractions appear varies from preparation to preparation; there is always an ethanol precipitate, but sometimes little or no material separates on the addition of $(NH_4)_2SO_4$. Residual protein is removed from these fractions, as from the pyridine fractions, by the Sevag technique. For effective removal of protein from these viscous solutions, the concentration must be kept low. Solutions of crude hyaluronic acid containing less than 2 g./l. at pH 4 in the presence of 0·1–0·5 M-salt are shaken briskly by hand with a mixture of one part chloroform to two of amyl alcohol, and then centrifuged. The aqueous solution is shaken again, this

time mechanically, with fresh solvent. As a rule little or no emulsion is made on this second shaking. After acidification with HCl the fluid can be either dialyzed directly or precipitated with 1·33 vol. of ethanol and then dialyzed in a smaller volume of water. In the latter case precipitation will be incomplete, but this is an advantage because the unprecipitated material has a lower viscosity than that precipitated. It can be recovered as an interfacial precipitate by adding $(NH_4)_2SO_4$.

The yield of purified material, by either of the two methods, is 1–2 % of the dry weight of the cords. The cord residue and the mucin clots that have been precipitated by adding acid to the extracts contain more hyaluronic acid, and this can be recovered, in a less viscous form, by digestion with proteolytic enzymes. The cord residue, of a consistency such that little more can be squeezed out of it by hand, is suspended evenly in about 5 vol. of 0·01 N-HCl containing 0·5 g./l. pepsin. The pH is readjusted to 2 if it is significantly higher than this, and the mixture is incubated with toluene for 24 hr. at 37°. The greater part dissolves leaving a pink solution and a curd. These are easily separated by filtration on a coarse paper at room temperature. Sufficient gelatin is liberated into the fluid to
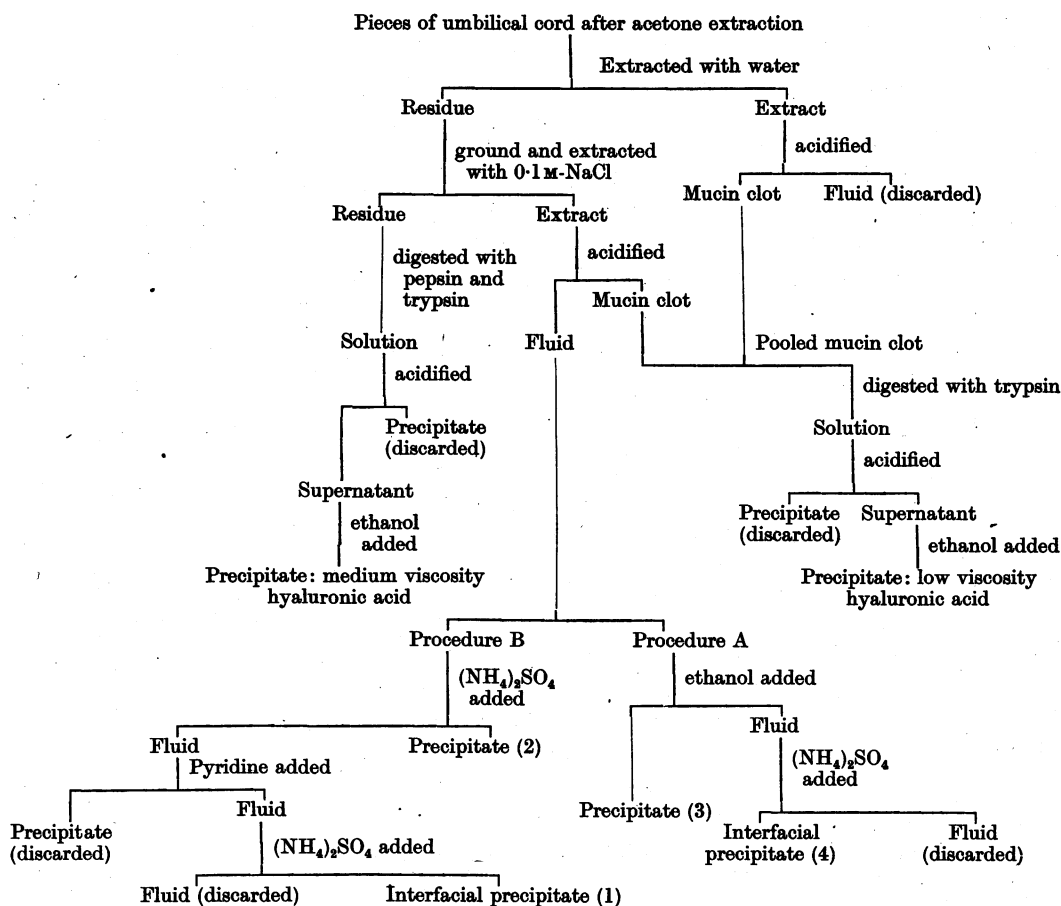
Fig. 1. Processes used in the extraction of hyaluronic acid in different forms. Precipitates (1), (2), (3) and (4) are shaken with chloroform and amyl alcohol to give hyaluronic acid fractions with variable but high viscosities.

cause gelatinization if it is cooled. This pepsin extract gives an ethanol precipitate and an interfacial precipitate in much the same way as the extract from the minced cords, but each has a high nitrogen content. It is preferable therefore to neutralize the pepsin extract and digest it with trypsin to destroy the protein that it contains. The cord residue is also digested with trypsin and the two extracts are combined. These digestions are carried out at 37° in the presence of toluene with 2 g./l. commercial trypsin for 24 hr. at pH 7·6. When these trypsin-digested fluids are put through the acid-ethanol-$(NH_4)_2SO_4$ fractionation, nothing precipitates with the conc. HCl and very little interfacial material separates. What there is, therefore, is added to the ethanol precipitate and this precipitate is dissolved, reprecipitated, and dialyzed.

The mucin clot can be partially fractionated by precipitation with ethanolic potassium acetate in alkaline solution as in McClean's (1943) method of purification, but it is generally more satisfactory to incubate it with trypsin and then work up the hyaluronic acid by ethanol precipitation in the manner just outlined for the pepsin extract of the cord residue. The yields of these two products are variable, but that from the pepsin extract may be as high as 4%, and that from the mucin clots 1%, of the dry weight of the cords. It is clear, therefore, that our methods for the preparation of the viscous fractions of hyaluronic acid lead to incomplete extraction in the first place and to significant losses during the purification. This state of affairs seems to be unavoidable if viscous preparations are desired. Material from the pepsin extract has only half the viscosity increment of the viscous fractions and that from the mucin clots is even less viscous. Slightly more viscous products can be made from these by precipitating a series of fractions with ethanol or ethanolic potassium acetate, but no product has been made from them at all resembling the viscous fractions. In Fig. 1 an outline is given of the processes used in making these various fractions, and also of the sequence in which they occur.

Properties of preparations of hyaluronic acid
General

Umbilical cords that have been washed with acetone and water and dried contain 2·5–3 % of amino sugar estimated as glucosamine by the method of Elson & Morgan (1933). This amount of glucosamine would correspond to 6–8 % of hyaluronic acid, which is equal to the total yield that we get. The non-viscous fractions however contain less than the theoretical percentage of glucosamine. Some of the glucosamine is therefore unaccounted for, but it is clear that the greater part of the glucosamine of the cord is present in the form of polysaccharides that can be isolated by the methods described here.

Preparations made from the acid-soluble part of the original cord extract are all clear and colourless, those made by digesting the mucin clot or the cord residue are yellow or pink. The nitrogen content (Kjeldahl) lies in the range 2·8–4·3 %. The phosphorus content is lower than 0·05 %, but sulphur contents of 0·5–1 % are usual. The form in which

this sulphur occurs is not known. Viscous preparations contain between 9 and 11 % of acetyl, but preparations made by enzymic digestion of the residues have lower acetyl contents, sometimes as low as 6 %. The acetyl content was measured by hydrolyzing a sample for 75 min. at 100° in 2·5 N-H_2SO_4, steam distilling in the apparatus described by Markham (1942) and titrating the distillate with N/70 NaOH. There is a similar deficiency in the glucosamine content; non-viscous preparations contain 20–25 %, whereas viscous preparations contain 34–38 %. These values are in the range found by most other workers. Glucosamine determinations were made by the Elson & Morgan (1933) technique on material that had been hydrolyzed for 6–8 hr. at 100° with 5 N-HCl and then evaporated to dryness in a vacuum desiccator over NaOH. These analyses were made on portions of dialyzed solutions, the concentration of which was determined by drying at 100° for 1–2 hr., and cooling in a desiccator. They refer to the free acid and not to a salt.

Viscosity

Viscosity measurements were made in Ostwald viscosimeters with capillaries 9 cm. long and having flow times of about 30 sec. for 4 ml. of water. This volume was used in all the experiments reported here and, except where another temperature is specified, all measurements were made at 25°. Viscosity measurements made in the absence of salt are of little significance; the large influence exerted by low concentrations of salt makes such a measurement an index of the completeness of dialysis rather than of the state of aggregation of the hyaluronic acid. From a consideration of the results of Madinaveitia & Quibell (1940) and of our own results we have adopted 0·05 M-NaCl and 0·05 M-phosphate buffer at pH 7·0 as the standard ionic environment for measurements. Small variations in salt concentration in this region have little effect on the viscosity. Much of the work on hyaluronic acid has been done at pH 7, and we adopted this pH because we intended to use it in subsequent enzyme studies. A more acid pH would be in some ways preferable, for the viscosity of hyaluronic acid in 0·025 M-citrate buffer and 0·15 M-NaCl, is unaffected by the pH in the range 4·5–7·3. There is a sharp fall in viscosity to about half the maximum between pH 4·5 and 2·6. Above pH 7·3 there is a more gradual fall to 95 % of the maximum value at pH 8·5.

Pyridine precipitates, before the removal of residual protein by shaking with chloroform and amyl alcohol, are the most viscous products that we have encountered. Solutions containing 0·3 g./l. have a relative viscosity in salt of 2·2–2·5, but this falls to 1·9–2·2 when the protein is removed. The results of Blix & Snellman (1945) suggest that this may be due to atmospheric oxidation during the shaking; it

might be avoided, as they suggest, by shaking in nitrogen but we have not tried this. The three other viscous products, after removal of protein, have relative viscosities of 1·7–2·0 for the 0·3 g./l. solution. In these cases the removal of protein may be accompanied by either a rise or a fall in viscosity. Occasional batches have had viscosities lower than this, and there is a gradual fall in viscosity as solutions age. Products from the cord residue have relative viscosities of 1·3–1·5 at 0·3 g./l. and those from the mucin clot 1·01–1·15. McClean & Hale (1941) and Blix & Snellman (1945) have published data on the non-linearity of the relationship between viscosity and the concentration of hyaluronic acid solutions. Our results are in agreement; with viscous preparations an increase in the concentration from 0·3 to 0·6 g./l. increases the viscosity increment three- to fourfold, and solutions containing 1 g./l. have a relative viscosity of more than 8. With less viscous preparations the relationship is practically linear.

Effects of temperature on the viscosity

Differences in the temperature at which the measurements were made must contribute to the differences in the results collected in Table 1. The flow times of 0·3 g./l. solutions of preparations covering a range of viscosities have been measured at various temperatures in the salt mixture already specified. The flow time of all is reduced by approximately one third between 20 and 37°, but much of this reduction is due to the change in the viscosity of the buffer solution. Over this temperature range the change in viscosity increment is never more than 15 % of the value at 20°. Temperature is clearly not a significant factor in explaining the differences between products made in different laboratories.

Effect of different salts on the viscosity

Madinaveitia & Quibell (1940) and McClean & Hale (1941) have shown that the viscosity of hyaluronic acid depends on the ionic environment. We have tried the effect of several salts on different preparations, and the results shown in Fig. 2 are typical of those given by viscous preparations from which the protein had been removed by the Sevag technique. An abrupt fall in viscosity with traces of salt is followed by a more gradual fall to a

minimum viscosity at about 0·5 M. There is then a gradual rise in relative viscosity; the values at 1 M are approximately the same as those at 0·01 M.
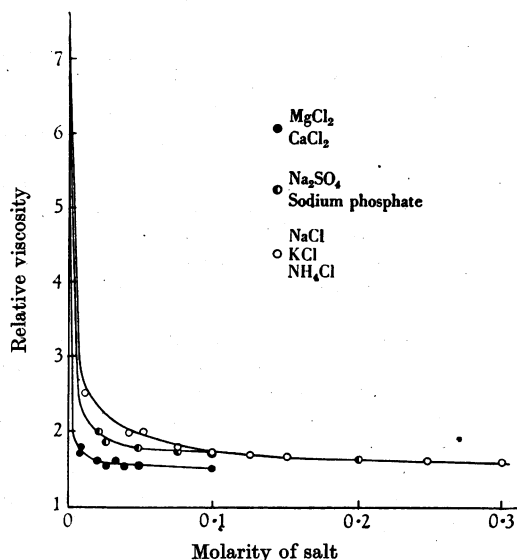

Fig. 2. Change in the viscosity of hyaluronic acid solution with varying concentrations of different salts. Temp. 25°; pH 7; 0·3 g./l. solution of a medium-viscous preparation.

Calcium and magnesium chlorides have clearly a greater effect than the other salts, but no generalizations can be made on the basis of such a small number of salts.

SUMMARY

1. Methods are described for preparing hyaluronic acid from human umbilical cords.

2. By preparing a series of fractions some products have been made with a greater viscosity than any hitherto described.

3. Some data on the chemical and physical properties of these fractions are given.

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