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THE BIOLOGICAL SIGNIFICANCE OF HYALURONIC ACID AND HYALURONIDASE

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Hyaluronic acid is a mucopolysaccharide acid which in animal tissues seems to bind water in interstitial spaces. It further holds cells together in a jelly-like matrix and serves as a lubricant and shock-absorber in joints. It is desaggregated and depolymerized by the action of the enzyme hyaluronidase. In this review the effort is being made to discuss aspects most essential to an understanding of the biological rôle of hyaluronic acid and hyaluronidase.

Duran-Reynals in 1942 in a paper entitled "Tissue permeability and the spreading factors in infection" published the first comprehensive review dealing with hyaluronic acid, hyaluronidase and the spreading factors. Current literature has been briefly reviewed in 1942 (15B) and in 1944 (44A).

I. HYALURONIC ACID. A. *Occurrence.* Hyaluronic acid was first isolated from bovine vitreous humor (63). Subsequently it was isolated from human umbilical cord (64), from bovine aqueous humor and from vitreous humor of pig eyes (65), from groups A and C hemolytic streptococci (35), from bovine and human synovial fluid (66), from a number of mesenchymal tumors like fowl leucosis (33), the pleura and peritoneal fluid of a case of mesenthelioma (55), from two cases of human synovioma (unpublished), from Rous and Fuyinami tumors of chicken (71), and from pig skin (56).

Hyaluronic acid probably occurs in the nucleus pulposus of the intervertebral disc (unpublished) and in small concentrations in connective tissue, although it has not been isolated from these sources.

B. *Chemistry.* For the isolation of the acid from various sources some modifications of the same general principles have been described. From fluids like vitreous humor, synovial fluid and some tumor fluids, from which the purest samples have been obtained, the acid is first precipitated as a salt of accompanying proteins. For this purpose the fluids are diluted with 2–5 volumes of cold water and acidified to a pH of about 4 with 50 per cent acetic acid with vigorous stirring. With vitreous humor the precipitation may be incomplete until the protein content is increased by addition of horse or cattle serum. The "mucin clot" is left standing at 0° for 24 hours, is then washed with cold water and extracted with a 5 to 10 per cent solution of sodium, potassium, or calcium acetate, depending upon which salt is to be desired. The extraction may be hastened by adding alkali to a pH of 9.0.

Solid tissues like umbilical cord or some tumors are chilled, ground and desiccated with acetone. The dry material is extracted repeatedly with 5 to 10 per cent of an acetate. The crude acid is acidified to pH 4 and precipitated with 1.5

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volume of ethanol. The centrifuged and washed precipitate is again extracted with acetate.

The further purification of this extract and the extracts of the protein salts described above are, from here on, identical. The bulk of the protein is removed by stirring with a chloroform-amylalcohol mixture for one hour followed by separation of the fluid from the gel by centrifugation. The procedure is repeated until a gel is no longer formed. Further nitrogenous impurities are removed either by adsorption on $Zn(OH)_2$ at pH 7.2 (adding 10 per cent zinc acetate and neutralizing) or by adsorption on Lloyd's reagent at a pH of about 4. The supernatant is precipitated with 1.5 volumes of ethanol at pH 4. The stringy mass is again centrifuged, washed with alcohol, redissolved in a minimum amount of water, precipitated by alcohol in the presence of acetate, washed with alcohol and ether and dried in vacuo over P_2O_5. In earlier preparations in this laboratory, the free acid was precipitated by pouring the aqueous solution into 6 to 10 volumes of glacial acetic acid. The products so obtained were of high purity and very low ash content (64), but were somewhat depolymerized as evidenced by a lowered viscosity and a lower optic rotation.

The hyaluronate preparations sometimes contain nitrogenous impurities, glycogen, and organic and inorganic sulfate. Nitrogenous impurities shown by an increased ratio of total N to hexosamine N can be removed by repeated adsorption. Glycogen, present especially in skin and umbilical cord preparations, is indicated by too low analytical figures (see below) and is removed by incubation for a few hours with either filtered saliva or a commercial amylase. Sulfates are difficult to remove, especially in umbilical cord hyaluronate. The S content may be lowered by dissolving the purified material in 10 per cent calcium acetate or chloride and fractionating with 25 to 33 per cent ethanol. After the mixture has stood at 0° for 24 hours the bulk of the sulfate is found in the precipitate.

Several authors have reported simplified methods for the preparation of hyaluronate. One such method (18) yielded a preparation which after removal of some insoluble material contained only 16 per cent of standard hyaluronate.

The preparations in this laboratory are analyzed for N by the Kjeldahl method, for hexosamine, for acetyl by the Kuhn-Roth method, for uronic acid by the Freudenberg modification of the Tollens-Lefevre method, and for ash.

The rotation $[\alpha]_D^{20}$ of the sodium salt is −70°, that of the free acid usually is somewhat lower, depending on the time of contact with acetic acid. The viscosity relative to 0.9 per cent sodium chloride at 37° of a 0.3 per cent solution of the sodium salt usually is approximately 4, and varies with the source. From vitreous humor by fractionation with $(NH_4)_2SO_4$ at a pH of about 9.0, fractions of widely varying viscosities were obtained. This is probably due to enzymatic depolymerization in vivo. The acid is poly-disperse in other sources as well, as pointed out by Blix and Snellman (4), who measured viscosities and streaming double refraction of flow. The latter was positive for both hyaluronate and chondroitin sulfate of cartilage. The particle length of different preparations of hyaluronate varied between 4800A° and 10,000A° with the higher values

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in preparations of umbilical cord. The corresponding molecular weights were estimated to be in the order of 200,000 to 500,000 with the assumption of an unbranched chain and a length of 10A° of the basic disaccharide unit. The molecular weights, according to the authors, should be regarded as minimal values.

X-ray measurements of sodium hyaluronate gave only diffuse patterns in powder and fibre diagrams.

The viscosity of isolated hyaluronate is much smaller than that of the fluids from which it is obtained. Whether this is due to oxidative breakdown, as suggested by some authors (83), or to another process of disaggregation or depolymerization cannot be decided with certainty. In the native state hyaluronate forms gels of varying rigidity. For example a cyst formed by an undiagnosed malignant tumor of the bone had the consistency of a 4 per cent agar solution. The cyst content was liquefied rapidly by incubation with testicular hyaluronidase. Samples of human synovial fluid exhibit viscosities ranging from very low values to high viscosities which are outside the range of our viscosimeters. The dilution curve of the tumor fluids showed a sharp drop in viscosity with dilution (55) and the same has been shown by Ragan (72) for normal human joint fluid. Obviously the viscosity in these fluids is of a non-Newtonian type. On the other hand, isolated hyaluronate has all the characteristics of a highly asymmetrical molecule. Aqueous solutions have high viscosity, they possess bi-refringence of flow, and can be spun into threads of considerable tensile strength.

The difference between the viscosity of the native fluid and that of isolated hyaluronate may be due to complex formation with accompanying protein, but there is no experimental proof for this. The mobility of isolated hyaluronate in the Tiselius apparatus at pH 7.8 was identical with that of the fast component in tumor and synovial fluids (55, 3). No fraction with a mobility intermediate between protein and polysaccharide was observed. Furthermore, on incubation of the native fluids with hyaluronidase, the viscosities drop rapidly to the values for the proteins present. It is concluded from these experiments that hyaluronate exists in these fluids in varying degree of polymerization and aggregation, the latter being due to weak secondary bonds either between the polysaccharide molecules themselves or between protein and polysaccharide. The weak bonds are broken by the processes involved in the isolation.

The chemical structure of hyaluronic acid is unknown. Equimolar quantities of hexosamine, acetyl and uronic acid have been found by analysis. Glucosamine has been isolated (as the crystalline hydrochloride) in yields up to 95 per cent of the colorimetrically determined amount. The presence of glucuronic acid was established by oxidation of hyaluronic acid by HNO_3 and isolation of saccharic acid as the crystalline acid potassium salt. From an enzymatic digest of synovial hyaluronate with pneumococcus hyaluronidase, glucuronic acid has been isolated as thiosemicarbazone (66). Hyaluronic acid, in contrast to chondroitin sulfate, probably contains little or no branched chains. This is indicated by the almost quantitative hydrolysis by some hyaluronidase preparations. It can be deduced from enzymatic data (see later) that the

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basic unit is a disaccharide with a free aldehyde group present in the acetylglucosamine moiety.

The occurrence of ester linkages in the hyaluronate chains has been recently suggested (4), on the basis of the alkali sensitivity of the polysaccharide. In 4–5 days in absence of O_2 at 20° about one milliequivalent of acid was produced for about every 4 disaccharide units. Apparently the reaction came to about the same endpoint with different samples of the polysaccharide and with varying alkali concentrations. About 10 per cent of the weight of the polysaccharide dialyzed through a cellophane membrane and the rest was clearly not of uniform size. The analysis for sodium in the neutral native material did not support the presence of ester linkages. Sensitivity to alkali with production of acidic groups with fission of the chains is common to many other polysaccharides, for example cellulose, though the mechanism is not clearly understood.

Hyaluronate on injection into animals is not antigenic. Attempts to confer antigenicity to hyaluronate have been unsuccessful (29).

II. HYALURONIDASE. A. *Occurrence.* The enzyme hydrolyzing hyaluronic acid, hyaluronidase, was first found in autolysates of a rough type II pneumococcus (58). Its occurrence was shown further in other types of pneumococci both smooth and rough, in strains of hemolytic streptococci of groups A and C, and in some anaerobes (62). Its occurrence in the animal body in extracts of spleen and of ciliary body and iris, has been reported (62).

The very wide distribution of hyaluronidase, however, was found after Chain and Duthie (8) discovered that the hyaluronidase activity of testicular extracts paralleled the activity of the "spreading factor", reported earlier by Duran-Reynals (27) and by McClean (46). Hyaluronidase activity, as measured by the hydrolysis or depolymerization of hyaluronic acid and hyaluronate-containing fluids, was then demonstrated in virulent and avirulent strains of type I pneumococcus (57), in a group C hemolytic streptococcus and in rabbit skin (ibid), in the venoms of many poisonous snakes, like copperhead, Black Tiger (9), rattlesnake (15), in bee venom (9), in Staph. aureus, Cl. Welchii, (9), and others.

B. *Determination of Hyaluronidase.* For the determination of hyaluronidase biological, chemical and physico-chemical methods have been used.

1. *Biological methods.* The spreading reaction has been reviewed by Duran-Reynals (12) and need be mentioned here only briefly. India ink probably is the best indicator, since with it the area of spreading is more circumscribed than with diffusible dyes. The most convenient dye is the blue dye T1824, since it is non-toxic and available in sterilized isotonic solution. An improved quantitative method for the estimation of the spreading reaction has been published by Humphrey (28) who injected the spreading agent intracutaneously into groups of 6 guinea pigs. The animals were killed 20 minutes after injection, the skin was removed immediately and the size of the bleb measured at the inner surface of the skin. The minimal diffusion dose is the least amount of enzyme which will produce a 20 per cent increase of the mean area over the mean area of the controls.

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The spreading reaction cannot be considered as an accurate assay of hyaluronidase. Some unspecific agents give spreading reactions. Furthermore, the correlation between spreading reaction and physico-chemical methods of hyaluronidase estimation is poor. (See later.) The influence of pressure on the assay of hyaluronidase by the spreading reaction has been stressed in a recent article (23A). Most investigators however have in the past used a constant and small volume of 0.1 or 0.2 cc. Control injections with identical volumes have probably been used by all investigators. Humphrey has emphasized the importance of the rate of the initial reaction by using a reaction time of only 20 minutes.

The decapsulation of mucoid hemolytic streptococci of groups A and C has been compared with hyaluronidase activity estimated by other methods (47). However, these organisms lose their capsules under various conditions without added enzyme. It is not known whether or not this is due to hyaluronidase produced by the same cultures (see later). The decapsulation of streptococci can hardly be considered as a convenient method for hyaluronidase estimation.

2. *Physico-chemical methods.* Three methods will be discussed: *a*, the mucin clot prevention test (abbreviated M.C.P.); *b*, the viscosity reducing method, and *c*, the turbidimetric method.

a. This method is based on the observation that native hyaluronic acid in acid solution coprecipitates with protein in a typical fibrous "mucin" clot. After incubation with hyaluronidase the quantity of the clot is reduced and the character of the precipitate changes from a fibrous to a flocculent precipitate, until finally the solutions remain clear. The test was apparently first used by Robertson, Ropes and Bauer (76) and has been modified by McClean and his collaborators (52).

The test is simple and suited for serial determinations and is probably the most sensitive test for low concentrations of hyaluronidase. However, it is positive only with crude hyaluronate, the mechanism of its action is not clear and it has a very poor correlation with other tests. The M.C.P. test is carried out in the native fluid or the isolated and redissolved protein salt. Pure hyaluronate precipitates protein in acid solution in flocculent form. (The difference between native and isolated hyaluronate has been discussed on p. 337.)

The error of the M.C.P test has been estimated (48) as ±25 per cent. The author found no correlation between M.C.P. units and viscosity reducing units, nor a constant trend with the potency or the source of the enzyme. Similarly in another paper from the same laboratory (77) the ratios of M.C.P. over viscosity reducing units with different micro-organisms varied between 1:20 to 1:162. The reason for the discrepancy between the two tests cannot be entirely the existence of different hyaluronidases in different sources (see later).

b. The viscosity reducing method. Advantages of this method are its accuracy and its simple kinetics. It has the disadvantage of being cumbersome and not easily carried out in serial experiments. The first systematic use of this method was published by Madinaveitia and Quibell (41) with a crude extract of acetone precipitated vitreous humor as substrate. The time required to reach half

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viscosity was found to be inversely proportional to the concentration of enzyme. Details as to substrate and sodium chloride concentrations, pH and temperature vary somewhat in different laboratories. The procedure used in this institute is as follows: 5 cc of a 0.3 per cent solution of pure sodium hyaluronate in 0.1 mol acetate buffer of pH 6.0 containing 0.15 mol sodium chloride are warmed to 37° for about 5 minutes. One cc. of enzyme dissolved in the above buffer mixture is added, and 5 cc. of the mixture are immediately transferred to an Ostwald viscosimeter in a constant temperature bath of 37.0°. The viscosities are determined repeatedly, until less than half viscosity of the mixture is reached. One unit is defined as the amount of enzyme required to reach half viscosity in 30 minutes. The viscosity of the substrate mixture in the control is about 4.0 relative to the solvent. The viscosity of the substrate remains constant for at least 2 weeks at 4° without preservative.

c. The turbidimetric method. This method, described first by Kass and Seastone (34), is based on the observation that pure hyaluronate at pH 4.2 gives a fairly stable colloidal suspension with dilute serum, whereas depolymerized hyaluronate remains clear. The method is reproducible to about 10 per cent, requires little substrate and is readily applied in large series. In a modification developed in this laboratory (21) the time of incubation is kept constant; in the original procedure the time is varied, the enzyme concentration being constant. In this modification the reaction is carried out in the presence of sodium chloride. Dilution of the enzyme in 1 cc. volumes of 0.1 M acetate buffer of pH 6.0 is warmed to 37° for 5 minutes. To each tube 1 cc. of the substrate is added, containing 4 mgm. per cc. of sodium hyaluronate in M/10 acetate of pH 6.0 and 0.3 mol sodium chloride. The mixtures are incubated for 30 minutes at 37°, immersed in a 60° water bath for 10 minutes to inactivate the enzyme, and 1 cc. aliquots are pipetted into test tubes containing 3.0 cc. of 0.5 M acetate of pH 4.2 and 1 cc. of acidified horse serum and mixed. The serum solution is prepared by 10 fold dilution of the sterile normal horse serum (containing no preservative) with 0.5 mol acetate buffer of pH 4.2 and acidifying to pH 3.1 with 4 N hydrochloric acid. The turbidities are read after 30 minutes at room temperature in a Coleman spectrophotometer using a wavelength of 580 mμ. One unit is defined as the amount of enzyme which reduces the turbidity given by 0.2 mgm. of hyaluronate to that given by 0.1 mgm. The values are read from a standard curve. The latter has been checked with 10 different preparations of hyaluronate from different sources. All points fall on the same S shaped curve. In the region between 0.05 and 0.15 mgm. the curve is almost a straight line.

The ratio of the units determined viscosimetrically to those determined turbidimetrically with 4 different hyaluronidase preparations varied only from 1.2 to 1.4. In this comparison the conditions of the two tests were identical with the exception of the substrate concentration.

3. *Chemical methods.* Chemically hyaluronidase activity has been determined by measuring the increase in reducing sugar, or by the increase in liberated acetylglucosamine. In both methods pure hyaluronate of known hexosamine and uronic acid content should be used. The main disadvantage of the reducto-

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metric method is its complicated kinetics, obviously the resultant of the action of different enzymes, which simultaneously split glucosidic linkages (see later). In this laboratory the method has been used extensively in the identification of hyaluronate from different sources. Either the Hagedorn-Jensen or the ceric sulfate method in the modification of Miller and Van Slyke was used and the results calculated as per cent of total reducing sugar expressed as equivalents of glucose. The substrate concentration usually was 0.5 per cent.

The increase in acetylhexosamine (68) has been used by some investigators (9) using synthetic acetylglucosamine as standard. It was pointed out that this method with the pneumococcal hyaluronidase indicated freeing of acetylhexosamine in excess of 6 times of the total weight of polysaccharide present (57). A similar result was reported by Hahn for testicular and Cl. perfringens hyaluronidase (19). Whether the measured rate of the color reaction of the enzymatic hydrolysis over that of synthetic N-acetylglucosamine is due to a structural difference in the acetylglucosamine or due to the linkage to the uronic acid in the hydrolysis product has not been decided.

In a recent paper Humphrey (31) confirmed the discrepancy between reducing and acetylhexosamine values on hydrolysis of hyaluronate with enzymes of bull testis, Cl. Welchii, and two strains of streptococci. The excess of the acetylhexosamine varied with different enzyme preparations and was not as high as found in this laboratory with pneumococcal enzyme. He further reported that the enzymic hydrolysates gave a direct colour in acid solution with Ehrlich's reagent without preliminary treatment with alkali, which is necessary to form an oxazoline derivative from acetylglucosamine. The author discusses whether oxazoline rings are preformed in the hyaluronate molecule. The alternative that enzymatically formed acetylglucosamine is labile and easily condenses to a heterocyclic compound seems more probable. The hydrolysis of chondroitin sulfate by testicular enzyme showed no excess of acetylhexosamine over the reducing values (32).

The method obviously cannot be used for the determination of hyaluronidase activity.

C. *The Mechanism of Hyaluronate Degradation by Hyaluronidases.* The dual nature of the two glucosidic linkages in hyaluronic acid, one belonging to the N-acetylglucosamine, the other to the glucuronic acid moiety, suggests that the depolymerization and the hydrolysis into monosaccharides require two enzymes. A comparison of hyaluronidases of different origin, measured viscosimetrically and reductometrically, indicated that hyaluronidases were mixtures of at least two enzymes, one attacking the long chain molecules, the other hydrolysing the aldobionic acid units formed (57). This conclusion was based on the observation that pneumococcal hyaluronidase hydrolysed the substrate almost to 100 per cent of the theoretical amount whereas testicular hyaluronidase, which showed a much faster rate in the viscosimetric tests than the bacterial enzyme, hydrolyzed the substrate to only approximately 50 per cent. The testicular enzyme on prolonged hydrolysis exceeded the 50 per cent reduction, but the total reduction was considerably short of 100 per cent. The addition of pneumococcal

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hyaluronidase to the non-hydrolysed residue brought about complete hydrolysis, while addition of fresh testicular enzyme had a negligible effect.

A still greater discrepancy between reductometric and viscosimetric activity was found with leech enzyme (60). One preparation as a flavianate reached a maximum hydrolysis, measured reductometrically of 40 per cent of the theory. Yet the same enzyme preparation turbidimetrically assayed 330 units, that is, it was equal to the best testicular preparations we prepared. This preparation showed an interesting difference from other hyaluronidases in the turbidity test, being uninfluenced by the absence of sodium chloride. Hahn (19) working with a purified enzyme prepared from aqueous extracts of the heads of leeches reported a maximal hydrolysis of only 26 per cent of the theory. Other preparations of leech enzyme in this laboratory analysed reductometrically as high as 70 per cent of the theory. It seems quite obvious that in purification one activity is increased at the cost of the other. Another difference between two hyaluronidases, one from bull testes, the other from streptococci, has been reported by Rogers (78). This author investigated the stimulation of hyaluronidase production by streptococci on addition of hyaluronate to a simplified culture medium. If instead of hyaluronate a digest of hyaluronate obtained with streptococcal hyaluronidase, was added to the culture medium, no stimulation of hyaluronidase production took place. If, however, a digest of hyaluronate obtained with testicular hyaluronidase was added, the stimulation of hyaluronidase production was almost as great as with native hyaluronate. Furthermore, the diffusible fraction of the latter hydrolysate was inactive, while the activity remained in the non-diffusible fraction (unfortunately the hyaluronate in these experiments was rather impure). Recently, similar observations were reported on the hyaluronidase production of Cl. Welchii (79).

Other evidence for the existence of several enzymes was brought forward by Hahn (20) who claims to have separated two enzymes from testicular extracts. One enzyme, called mucopolysaccharidase (abbreviated M.P.) hydrolyses to the aldobionic acid stage, the other muco-oligosaccharidase, (abbreviated M.O.) hydrolyses the disaccharide to monosaccharides. Since the M.P. liberates acetylglucosamine during hydrolysis, the free aldehyde group must belong to the hexosamine moiety, therefore the M.O. was called a glucuronidase. The two enzymes were fractionated by $(NH_4)_2SO_4$ and lead acetate, the M.O. activity precipitating in the first fractions. M.P. activity was measured by a viscosimetric method, and M.O. activity by a reductometric method. The substrate of the latter was hyaluronate previously digested for 4 days with M.P. followed by dialysis, the concentrated non-diffusible residue being the substrate. There was no linear relation between the percentage of hydrolysis and the enzyme concentration. The total reducing values must have been quite small, since the substrate concentration was very low, only 0.5 mgm. glucose equivalent per cc., and a turnover of only 10 per cent was measured.

To this reviewer it seems highly desirable to test the presence of the M.O. with a well defined substrate. One would expect the disaccharide formed by the 4 day hydrolysis to be diffusible. There also remains the question of whether the M.O.

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can or cannot attack the glucuronidic linkage in the native hyaluronate. The hydrolysis of the linkage in the native chain presumably would also cause a decrease in viscosity, while the end groups with the two enzymes ought to be different.

One distinct enzyme, β-acetylglucosaminidase, present in crude testicular and in crotalus hyaluronidase, as well as in sources showing no hyaluronidase activity, does not seem to be involved in hyaluronidase activity (13). This enzyme was found by Helferich and Iloff (24) in emulsin of sweet almonds. The substrate used was β-phenylacetylglucosamine. The enzyme was separated from testicular hyaluronidase with $(NH_4)_2SO_4$, lead acetate or $CuSO_4$ or adsorption on kaolin. Hahn (20) likewise has reported good evidence for the separation of hyaluronidase from β-glucosaminidase activity.

In summary, hyaluronidases of different origin undoubtedly contain different enzymes, but their number and mode of action are not fully known.

D. *Influence of Environmental Factors on Hyaluronidase Activity.* 1. *Effect of pH.* The effect of pH on the rate of hyaluronidase activity varies with the source of the enzyme, the salt concentration and the test method. In the reductometric procedure testicular hyaluronidase has a double optimum, one at about pH 4.5, the other at about pH 5.7 which has been interpreted as another indication for the presence of two distinct enzymes (57). The pH optimum of pneumococcus and Cl. Welchii hyaluronidase was found in the reductometric procedure at 5.8 (62). By a viscosimetric method, McClean (48) found a marked dependency on the salt concentration of the activity at different pH levels. In M/60 buffer the optimum of testicular hyaluronidase was at pH 6.8, while in M/6 buffer, the optimum shifted to pH 5.0. A similar behaviour was found with Cl. Welchii enzyme. Moreover, at the pH optimum the activity of the enzyme was much greater in the more dilute buffer. The marked difference found may have been partly due to differences in chloride ion concentrations (see later), especially with hyaluronate of umbilical cord. With the turbidimetric method, the pH optimum of testicular hyaluronidase in M/10 acetate in presence of 0.15 M sodium chloride was found to be 6.0 (21).

2. *Effect of salts.* Robertson et al. (76) reported that dialyzed Cl. perfringens hyaluronidase had no effect on a dialyzed "synovial mucin." Addition of phosphate or other salts in increasing concentrations (up to 0.1 M) resulted in increasing activity. Madinaveitia and Quibell (42) reported a marked influence of salts, especially sodium chloride, on the activity of testicular hyaluronidase, as determined viscosimetrically. The optimum was between 0.07 and 0.17 mol sodium chloride.

In this laboratory the influence of salts, especially sodium chloride, was found to be much more marked with the turbidimetric and viscosimetric methods than with the reductometric. The same behaviour had been shown with lysozyme and its substrate (59). The influence of sodium chloride on the rate of depolymerization depended further on the source of the substrate. This is illustrated in the following table in which the same enzyme preparation, highly purified testicular hyaluronidase, has been tested.

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It can be seen from this table that the apparent activity of a sample varied from 100 to 167 units per mgm. The wide variation in apparent activity in the absence of NaCl should be noted. By the quantitative turbidimetric test the different hyaluronates precipitated an equal amount of serum protein. Analytically, they were identical within the limits of the errors of the methods. Quantitative determinations in 100 mgm. of the tumor preparations after ashing with HNO_3 showed only a trace of chloride. The chloride effect and the differences between the hyaluronate preparations seem to be due to the presence of competitive inhibitors, as described in the next chapter. It is evident from these experiments that the purity and origin of the substrates greatly influence the titres of enzyme preparations.

3. *Inhibitors of hyaluronidases.* McClean (47) reported the inhibitory action of heparin and chondroitin sulfate on the in vitro decapsulation of streptococci by testicular hyaluronidase. Three-tenths per cent of the pure NH_4 salt of heparin suppressed the decapsulation of an enzyme concentration equivalent to one viscosity reducing unit. From 0.001 to 0.01 per cent of the NH_4 salt further inhibited the action of one unit of hyaluronidase on 0.1 per cent hyaluronate. Hyaluronate partly depolymerized by precipitation with acetic acid likewise

TABLE 1

SOURCE OF HYALURONATE	NO NaCl; UNITS/MGM.	0.15 M NaCl; UNITS/MGM.
Umbilical cord	5	100
Tumor	67	167
Synovial fluid	67	100
Strep. C	25	125

inhibited the decapsulation, while hyaluronate depolymerized enzymatically was without inhibitory effect. Gastric mucin also inhibited the decapsulation, but Shiga-Kruse polysaccharide and a blood group A hapten had no effect. It should be noted that all inhibitors are or contain acid polysaccharides; the two neutral polysaccharides tested were without action.

In this laboratory the inhibiting activity of heparin on the hydrolysis of hyaluronate was tested and results are shown in table 2. Heparin desulfurated with oxalic acid-barium oxalate had no effect. The inhibition with chondroitin sulfate was considerably less than with heparin, the ratio between the two being approximately 1:100.

The inhibitory action of normal human and rabbit serum on pneumococci, Cl. Welchii and streptococcus hyaluronidase seems to have been reported first by this laboratory (26). The reductometrically determined activity of the first two was inhibited 25 to 50 per cent by normal sera, while with streptococcus hyaluronidase the inhibition was smaller. McClean (47) reported the inhibition of guinea pig, rabbit, sheep, horse, mouse and human serum on hyaluronidase prepared from bull, rabbit and mouse testes, from streptococcus, Cl. Welchii, and from viper and scorpion venom. The inhibitory activity of the sera from

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different species against any one enzyme showed considerable variation. The inhibitory action of the sera was associated with the pseudoglobulin fraction. McClean suggested that the inhibition was due to some polysaccharide fraction of the serum.

Meyer et al. (62) found the hydrolysis of free hyaluronate twice as fast as that of an equimolar concentration of a protein salt of the acid, prepared from synovial fluid. In experiments of Kass and Seastone (34) the reduction of the turbidity of a preformed horse serum-polysaccharide precipitate required 10 times the enzyme concentration than that of the polysaccharide alone. More recently Leonard and Kurzrok (36) reported that normal sera inhibit the liberation of tubal ova by testicular hyaluronidase (see later).

The inhibitory action of serum on hyaluronidase of animal and bacterial origin recently was the object of three papers (18). A complete discussion of all the experimental evidence presented and the conclusions drawn from them seems

TABLE 2

	UNITS/MGM.	% INACTIVATION
Hyaluronidase + Hyaluronic acid	83	
Hyaluronidase + Hyaluronic acid + NaCl	200	
Hyaluronidase + Hyaluronic acid + 0.040 mgm. heparin	3	96
Hyaluronidase + Hyaluronic acid + 0.040 mgm. heparin + NaCl	200	0
Hyaluronidase + Hyaluronic acid + 0.004 mgm. heparin	12	85
Hyaluronidase + Hyaluronic acid + 0.0004 mgm. heparin	40	52
Hyaluronidase + Hyaluronic acid + 0.00004 mgm. heparin	83	0

impossible in this review and does not seem warranted. The invasive agent, hyaluronidase, is counteracted by enzymes of the sera of different species, ranging from man to carp, which were said to destroy hyaluronidase. Crude bacterial and some animal hyaluronidases in turn contain other invasive enzymes, which counteract the defensive enzymes of the sera, which in turn are again counteracted by another serum enzyme and so forth.

Experimentally, hyaluronidase was incubated with serum in a salt buffer mixture and the solution mixed with a very impure hyaluronate of umbilical cord in the presence of phosphate. The hyaluronidase activity was measured viscosimetrically. Phosphate was found by Haas to inhibit the action of serum on hyaluronidase, while other salts, especially borate, promote it. The enzymatic nature of the inhibiting agent, in serum was concluded from the temperature sensitivity, the dependence on pH and the inactivation by heating. This evidence appears inconclusive. The activity of sera among comparable data in different tables is very variable. The rate of inactivation was reported to depend

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considerably on the quality of the polysaccharide (18, p. 78). (A sample of hyaluronate prepared according to Haas in this laboratory contained 16 per cent of sodium hyaluronate and over 50 per cent inorganic material.) Furthermore, the inhibition by human sera depends not only on concentration but likewise on purity of the hyaluronidase (21). That is, the protein impurities present in hyaluronidase likewise react with the serum. The inactivation by sera is not only prevented by phosphate, but at least in part reversed on further incubation. The inhibitory action of serum furthermore is so dependent on the milieu that it seems very difficult to draw conclusions from experiments in which the complex systems are further complicated by adding crude bacterial filtrates or snake venoms. To this reviewer the data presented do not seem essentially different from the inhibition by heparin and other substances, except that the inhibitors in serum are more thermolabile. The great dependency on milieu conditions is interpreted by this reviewer as being due to competitive reactions between various proteins among themselves and for the acid substrate. That hyaluronidase can be active in the presence of serum proteins is shown not only by its spreading reaction in the skin, but its action in pathological human joint fluid and other fluids and its in vivo activity on sedimentation rate of blood when injected intravenously.

4. *Antisera to hyaluronidases.* The inhibition of the spreading reaction by specific antisera has been discussed by Duran-Reynals (12). Various papers published since then support the previous conclusions that antisera to hyaluronidases suppress the in vivo and in vitro activities of hyaluronidases, but that they are strictly specific for the source of the enzyme. Thus McClean (48) finds no crossing over with antisera against Cl. Welchii to Cl. septicum. Streptococcal enzymes were found to be group, but not type specific. An antiserum against crude bovine testicular hyaluronidase neutralized bovine, but not mouse testis hyaluronidase. Highly purified testicular hyaluronidase, however, has never been obtained in antigenic form (49).

E. *Bacterial Hyaluronidases.* Increase in hyaluronidase production by the addition of hyaluronate to the medium was first reported by McClean and Hale (49) for Cl. Welchii. The stimulating effect of added hyaluronate was studied systematically by Rogers (77, 78). In groups A and C hemolytic streptococci and in two strains of Cl. Welchii enzyme production was proportional to the concentration of hyaluronate added and rose from a titre of 1:50 M.C.P. units to as high as 1:100000 units, when the pH was maintained at neutrality by 8.5 per cent sodium glycerophosphate. One of two strains of staph. aureus produced a titre of 1:3200 units, which was not increased by hyaluronate, the other had a low titre with or without added hyaluronate. The titre was not raised either in a strain of Cl. septicum (77, 78). In staph. aureus the hyaluronidase production was raised by addition of a papain digest of peptone to the medium, while in Cl. septicum it could be increased by some specially prepared peptone (79).

A systematic study of hyaluronidase production in relation to serological groups and types, to capsule formation and to the sources of the strains in hemoly-

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tic streptococci was published by Crowley (11). A total of 376 strains were tested, 308 of which belonged to group A. Of the latter, 64 strains, all belonging to types 4 and 22, showed hyaluronidase activity. Seventy-two and 96 per cent respectively of all types 4 or 22 examined were hyaluronidase producers. The titres varied from 50 to 1000 M.C.P. units/cc. Thirty-five out of 55 group C strains and all of 13 examined of group G strains of a variety of types showed hyaluronidase activity. All hyaluronidase positive strains were non-capsulated, but many of non-capsulated strains were hyaluronidase negative. Little correlation was found between hyaluronidase production and virulence. Of streptococcus strains isolated from 127 cases of scarlet fever, 27.6 per cent; of 136 cases of sore throat, 36.8 per cent showed hyaluronidase activity. Of the latter half of the strains belonged to groups C and G. Twenty and four-tenths per cent of hyaluronidase positive strains were isolated from wounds, burns or impetigo contagiosa and 23 per cent from healthy throats. Of 6 cases of puerperal fever, 3 were hyaluronidase producers, among them a group C strain isolated from the blood of a fatal case. However, in another case where the organism was isolated from the blood stream, it was capsulated and negative for hyaluronidase. In some cases highly contagious strains were isolated from contacts in different individuals. The organisms were capsulated and none produced hyaluronidase. The author concludes that there was no evidence for correlation of hyaluronidase production of streptococci with any particular type of infection, nor with virulence of streptococci for man.

Hyaluronidase production of streptococci as reported by Crowley merits some discussion, especially in regard to negative findings in non-encapsulated strains. It was reported from this laboratory (57) that streptococcal hyaluronidase, present in the medium as well as in purified preparations, showed a rapidly diminishing activity as compared to pneumococcal hyaluronidase. Furthermore, the same strains of both group A and C organisms varied greatly in hyaluronidase production from completely negative to high concentrations, although grown under apparently identical conditions. In animals the culture medium and purified samples gave a pronounced spreading reaction regardless of the in vitro titre of hyaluronidase activity. It was suggested that the enzyme may be inactivated, the inactivation being reversible in vivo, while no reversibility could be demonstrated in vitro. Hale (22) confirmed the anomalous behaviour of streptococcal hyaluronidase in the viscosimetric tests but only when carried out at pH 4.6. Furthermore, exposure of the enzymes of groups A and C streptococci to pH 4.6 before mixing with the substrate resulted in complete inactivation of the enzyme. At neutrality the enzyme showed a normal fall in viscosity proportional to the enzyme concentration. In our experiments the substrate was the sterile pleura fluid of a patient with mesenthelioma. The reaction was carried out at a pH of about 7.0. The reductometric tests were carried out at pH 5.8 with pure hyaluronic acid as the substrate. Inactivation of the enzymes was apparent with both methods. Whether or not the inactivation is enzymatic has not been determined.

In view of these experiences with streptococci it may be questioned whether

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failure to demonstrate hyaluronidase is synonymous with absence of the enzyme. A renewed study of hyaluronidase in streptococci seems indicated, not only in regard to hyaluronidase production, but also in regard to spreading activity, which in our experience is not confined to types 4 and 22 of group A organisms.

The correlation between capsule formation and hyaluronic acid production has been studied by Seastone (82) in a group C streptococcus. The evidence there given for the rôle of the hyaluronate capsule in the virulence of the infecting organism has been corroborated by Hirst (25), who protected mice by the intraperitoneal injection of leech extracts against fatal infection with a strain of group C streptococcus, while against a group A strain only feeble protection resulted. Blundell (5) obtained a greater mean survival time in mice infected with a group A organism, when treated with testicular hyaluronidase than without treatment. McClean (47) reported failure to protect either against groups A or C organisms with testicular hyaluronidase. Kass and Seastone (34) protected mice against 10 to 100 M.L.D. of groups A and C organisms by repeated injections of testicular hyaluronidase. No protection resulted from injection of inactivated hyaluronidase. The specificity for streptococcus was demonstrated by the failure to protect against the fatal infection with a type I pneumococcus. The failure of other authors to obtain this effect was explained by the time lag between injections of the enzyme, which allowed the organisms to regenerate their capsules. Kass and Seastone further studied the in vitro effect of hyaluronidase on streptococci. Phagocytosis of group A organisms by human leucocytes and the killing power of whole blood was greatly increased by incubation of the organisms with hyaluronidase. In controls with pneumococci no change resulted from incubation with hyaluronidase.

In *pneumococci* no correlation between the amount of hyaluronidase produced and the clinical virulence, or between enzyme production and the type of organism, was found (30). Type I organisms rarely produced the enzyme.

In *staphylococcus aureus* no correlation between enzyme production and virulence was reported by Boe (6). However, Schwabacher et al. (81) found among 654 coagulase positive strains of staphylococci 86.7 per cent producing both α-lysin and hyaluronidase, 6.9 per cent hyaluronidase positive and α-lysin negative, 4.4 per cent hyaluronidase negative and α-lysin positive and 2 per cent negative for both. None of the 160 coagulase negative strains produced either α-hemolysin or hyaluronidase. The author concludes that clinical virulence is associated fairly clearly with α-hemolysin production and to some extent with hyaluronidase production. What part hyaluronidase plays in determining the virulence of a strain of Staphylococcus was not clear.

In Cl. Welchii, McClean, Rogers and Williams (52) found 12 out of 32 strains produced hyaluronidase. Of these 12 strains 11 produced toxin and 10 were derived from clinical cases of gas gangrene. In Cl. septicum 20 out of 20 strains in vitro produced hyaluronidase. Of these 4 were derived from clinical cases of gas gangrene, 9 were stock laboratory strains and 7 were contaminants of "healthy" wounds. Seven out of 15 strains of Cl. oedematiens produced a low

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titre of hyaluronidase. Six of these were stock laboratory strains and one was derived from a toxemia in sheep. McClean et al. (52) proposed the determination of hyaluronidase and lecithinase for early diagnosis of gas gangrene in wound infections. Guinea pigs were infected intramuscularly with organisms of the gas gangrene group and the presence of the enzymes was determined in the muscle extracts and edema fluid. With Cl. Welchii as infective agent the muscle contained 2 M.C.P. units 2 hours after infection. With Cl. septicum the infection developed more slowly than with Cl. Welchii. Edema fluid was not obtained before 12 hours after infection, when it as well as the muscle did contain hyaluronidase in low concentrations. With Cl. oedematiens the infection proceeded still more slowly than with Cl. septicum. In some instances 12 hours after infection hyaluronidase was detected in the edema fluid, but since many strains did not produce hyaluronidase in vitro, this organism was the least satisfactory from the point of view of detection of enzymes in the body fluids. The authors further proposed to utilize specific antisera for the differential diagnosis of the infecting agents in combination with the enzyme determinations, as in positive tests only the specific antisera will inhibit the enzyme of the infecting agent (50).

Clinically the proposal of McClean et al. was tested by MacLennan (39) in 39 cases, among them 12 cases of clinical gas gangrene, the others from various infections or from non-infected wounds. Only in one case, infected with a streptococcus pyogenes, was a positive M.C.P. test obtained.

McClean and Rogers (51) ascribed the failure of MacLennan at least in part to the treatment of the clinical cases with antisera. In guinea pigs the administration of antisera inhibited and sometimes suppressed the appearance of hyaluronidase and lecithinase. They further tested whether the size of the infecting dose explained the difference between the animal experiments and clinical cases. A minimal infecting dose of organisms did not diminish the titre in the infected tissues of the guinea pig. They further studied a combination of infecting agents. Cl. histolyticum, which produced no hyaluronidase, caused death in combination with a streptococcus earlier than Cl. histolyticum alone. The strain of streptococcus used did not produce illness in the guinea pig either in presence or in absence of added hyaluronidase. A combination of Cl. histolyticum with the streptococcus and added hyaluronidase caused death even earlier than in the previous experiments.

MacLennan and associates (40) maintained in a later article that the determination of hyaluronidase and lecithinase was unsatisfactory as a diagnostic procedure.

F. *Substrates of Hyaluronidase.* The difference in the apparent activity of testicular hyaluronidase on hyaluronate of different sources has been discussed above. Highly purified testicular hyaluronidase has been stated to hydrolyze beside hyaluronate only two other mucopolysaccharides, one a monosulfuric acid ester of hyaluronic acid obtained from cornea (54), the other the chondroitin sulfate of hyaline cartilage (57) (43). The hydrolysis of chondroitin sulfate is

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of considerable interest, since compounds of the general composition of this acid occur beside hyaluronate, in mesodermal tissue, like umbilical cord (64) and skin (56) in concentrations about equal to that of hyaluronate. The chondroitin sulfate of skin differed in rotation from that of cartilage (56). The two acids were found, in collaboration with Dr. Z. Dische, to differ further in their resistence to alkali and considerably in the time-intensity curve of their reaction with carbazole. Moreover, chondroitin sulfate of skin, and to a lesser degree of umbilical cord, in contrast to that of cartilage were resistant to hydrolysis with testicular hyaluronidase.

Is chondroitin sulfate of cartilage a substrate of hyaluronidase, or is it hydrolysed by another enzyme copresent in the testicular preparations? The evidence points to the latter explanation. Chondroitin sulfate is not attacked by pneumococcal or streptococcal or leech hyaluronidases. While it might be argued that the bacterial enzymes are to a lesser degree true mucopolysaccharidases, this cannot be claimed for the leech enzyme. Furthermore, purified testicular hyaluronidase after precipitation by acetone lost most of its chondroitin sulfate hydrolysing power, while the hydrolysis of hyaluronate was hardly impaired. However, it seems remarkable that hyaluronidase activity towards hyaluronate runs practically parallel with the activity towards chondroitin sulfate, whether the fractionation is carried out with metal salts or with adsorption. The enzyme hydrolyses chondroitin sulfate with the production of a disaccharide without hydrolysing the sulfate linkage. Humphrey in a recent paper (32) found no hydrolysis of chondroitin sulfate with streptococcal enzyme and a negligible effect with an enzyme prepared from Cl. Welchii. Crude and a highly purified testicular enzyme however at pH 4.8 and 6.0 liberated reducing sugars with chondroitin sulfate at a rate comparable to hyaluronate. Some bacterial extracts have been reported to hydrolyse chondroitin sulfate by splitting off inorganic sulfate (69).

III. Hyaluronic acid and hyaluronidase in animal physiology and pathology. A. *Ocular Fluids.* Hyaluronic acid has been isolated from vitreous humor of cattle, swine, and sheep and from the aqueous humor of cattle. Its concentration in the normal fluids has been determined by analysis of the hexosamine content. This procedure involves only a small error, since the protein, being very low in these fluids, contributes very little to the total hexosamine. The concentration of hyaluronic acid found by isolation was in fair agreement with that calculated from the hexosamine values. The polysaccharide content of vitreous humor varies considerably with the species, from a low of about 9 mgm. per cent in the cat to a high of about 48 mgm. per cent in cattle eyes, while the concentrations in aqueous humor showed little variation (67).

The origin of the polysaccharide in the ocular fluids is not known with certainty. While the permanent structures of the vitreous body seem to be of retinal origin (44), the fluid part probably originates mainly in the ciliary epithelium which (in this country at least) is considered a secretory epithelium. The presence of hyaluronate in the fluids and its absence from serum have been cited as evidence for its origin from a secretion rather than a dialysate (65). Hyaluronate on injection into the anterior chamber of rabbits disappears rather

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rapidly. After paracentesis of the anterior chamber the acid reappears (as judged by analysis of the hexosamine) after the return of the protein content to normal. It was concluded that the acid was constantly produced and removed, presumably after enzymatic hydrolysis. Hyaluronidase has been reported in extracts of the ciliary body and iris (67) though Chain and Duthie (9) denied its occurrence in these tissues and suggested bacterial contamination as a source of the enzyme. The presence of hyaluronidase has been definitely established in pooled aqueous humor obtained by aspiration of the eyes of freshly killed cattle. The aqueous humor was lyophilised and the residue dissolved in 1/10 of the original volume. Four units (turbidimetric) of hyaluronidase were found per cc. of the concentrate, corresponding to 0.36 unit per cc. of the native fluid (21). It is of considerable interest that the hyaluronate present in the same sample was found to be almost completely depolymerized. While the vitreous humor of the same eyes had a hyaluronate concentration (turbidimetric) of 0.75 mgm. per cc., the lyophilized aqueous humor showed only 0.0015 mgm. per cc. By hexosamine estimation the hyaluronate concentration of the vitreous humor ranged from 0.32 to 0.52 mgm. per gram (the lower figures are probably explained by the difference in specific gravity and evaporation of water). In aqueous humor the hyaluronate concentration was about 0.03 mgm. per gram, a value in fair agreement with the concentration found by isolation of an impure fraction (0.046 mgm. per gram). Since the concentration by the turbidimetric method amounted only to 0.0015 mgm. per cc., we may conclude that the hyaluronate in aqueous humor is depolymerized to about 95 per cent of the total. As this depolymerization presumably occurred in vivo, we assume that hyaluronate is after enzymatic depolymerization constantly removed through the normal exit channels of the eye.

What part hyaluronate plays in the physiology and pathology of vitreous humor is unknown. It probably takes part in the maintenance of the turgor of the vitreous body. The effect of intravitreal injections of purified testicular hyaluronidase in rabbits is being studied by Dr. L. von Sallmann. Injections of about 100 turbidimetric units appear to liquefy the vitreous humor in vivo. The cause of simple glaucoma may well be explained by inhibition of hyaluronidase in the eye.

B. *Synovial Fluid.* Bauer et al. (1) have reviewed the physiology of synovial fluid. The view expressed in their paper that synovial fluid contains a glycoprotein instead of a dissociated mucopolysaccharide seems no longer tenable (see p. 337). Furthermore, the concept of the origin of synovial fluid as a dialysate to which the "mucin" is added by the passage of the fluid through the connective tissue does not seem probable. If such a mechanism existed then pleura and peritoneal fluid and lymph should likewise contain the "mucin" which is not demonstrably the case. From the appearance of a viscous fluid in tissue culture of synovial tissue (86) it may be concluded that hyaluronate is a secretory product of some cells of the synovial lining. This seems to be borne out by the isolation of hyaluronic acid from a synovioma, not only at the original site of the tumor, but in metastases in the liver (60).

The concentration of hyaluronic acid in normal human synovial fluid has not

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been determined by isolation (see below). In cattle synovial fluid obtained by aspiration of astragalo-tibial joints the concentration by isolation was found between 20 to 25 mgm. per cent (66). From knees of patients with rheumatoid arthritis the acid was isolated in 3 different samples in concentrations of 60, 132 and 206 mgm. per cent. However, it may be questioned whether cattle synovial fluid as collected, is normal and comparable to the fluid in human joints. Its viscosity is low compared to that of normal human fluid taken at autopsy or from living patients with no pathology of the joints (72). By extrapolation of the dilution curve of human synovial fluid the relative viscosity of the latter was estimated as greater than 180, whereas that of the cattle fluid was less than 10. The volume of the fluid of cattle taken immediately after slaughter is quite large, sometimes over 50 cc. It seems possible that due to long standing and trauma of the animals before killing the cattle fluid is actually a mixture of synovial and edema fluid.

The hyaluronic acid and protein contents and the viscosities of about 30 pathological fluids have been determined (74). The viscosity was variable and never higher than that of normal knee joints. The protein content was over 3 per cent, that of normal human and cattle fluid below 3 per cent. The viscosity practically was all due to hyaluronate, since on incubation with enzyme the relative viscosity fell to slightly above 1.0, corresponding to the viscosity of the remaining protein solution (66).

By the turbidimetric method the hyaluronic acid content varied from 80 to 270 mgm. per cent. No direct proportionality between viscosity and hyaluronate concentration was found. In normal human and in cattle synovial fluid the hyaluronic acid concentration could not be determined turbidimetrically by the above method. In contrast to the pathological fluids the normal fluids in the turbidimetric procedure are precipitated as a coherent fibrous clot while the pathological fluids with few exceptions precipitated in the form of a stable turbidity, as do vitreous humor and the tumor fluids. On incubation of the normal fluids with 0.01 unit of enzyme, far too little to decrease the apparent hyaluronate concentration, the clot formation of the normal fluids is prevented and hyaluronic acid can be determined turbidimetrically. The amount found varied between 80 and 150 mgm. per cent as compared to 80 to 270 mgm. per cent in pathological fluids. That is, both the concentration and especially the total amount of the fluid in pathological joints is larger than that found in normal joints. Hyaluronidase in contrast to normal ocular fluid could not be demonstrated in synovial fluid, although the absence of fibre formation may suggest its presence in low concentrations in pathological joint fluids.

C. *Hyaluronic Acid in Tissues.* The presence of hyaluronate in tissues when isolation is not possible, can be made probable enzymatically by the use of a variety of bacterial and animal enzymes. Judging from the failure of isolation experiments and the absence of any effect on capillary permeability by purified hyaluronidase, hyaluronic acid is not present in capillary cement. The presence of hyaluronic acid in all connective tissues has not been established. Positive spreading reactions in stomach and intestinal walls, in the uterus, in striated

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muscle, fasciae and tendons with crude testicular extracts have been reported (12, 14). It remains to be determined whether the spreading is due to depolymerization of hyaluronate or chondroitin sulfate. In calves' tendon the polysaccharide is a sulfate ester and is hydrolyzed by testicular hyaluronidase at a fast rate (60). Only in skin and in umbilical cord do larger quantities of hyaluronate occur. It seems remarkable that in both tissues chondroitin sulfate is present in a concentration roughly equal to that of hyaluronate. Both substances have to be regarded as constituting essential components of the interfibrillar ground or cement substances in these tissues. It seems of great importance to gain more information about the nature of such cement substances especially for the understanding of the mechanism of diseases of the mesenchymal tissues as well as of wound healing and the problem of ageing.

Beside hyaluronate and chondroitin sulfate the mucopolysaccharide isolated from amyloid tissue may likewise be such a cement substance. This substance is a monosulfuric acid ester, containing acetylhexosamine and a uronic acid in equimolar proportion. The hexosamine has been isolated and characterized as d-glucosamine (60). The behaviour of the mucopolysaccharide towards hyaluronidases shows that it is not derived from hyaluronic acid. From its enzymatic behaviour and its reactions with alkali and with carbazole it appears to be closely related to heparin. It may well be possible that this substance accumulates in excessive amounts in certain pathological conditions, while in small concentrations it may be a normal component of some mesenchymal tissues.

Histologically the presence of hyaluronic acid has not been demonstrated, since no staining method was known. Hale (23) recently proposed a histochemical test for hyaluronic acid. After a specific fixation of the tissue, slices are treated with iron hydroxide. The iron combines with the acid polysaccharides. The bound iron is stained as Prussian blue. For the differentiation between hyaluronate and other polysaccharides incubation of the tissue slices with streptococcal hyaluronidase is recommended, since this enzyme does not attack chondroitin sulfate. No experimental results with this method have been reported.

Highly polymerized hyaluronate in a concentration of about 1 per cent shows in smears typical metachromatic staining with toluidine blue, while lower concentrations failed to show the effect. The metachromasia is prevented on incubation with hyaluronidase (unpublished experiments). The concentrations present in tissues are probably never high enough to contribute to the metachromatic staining. Therefore, the metachromasia of tissues, done under appropriate conditions, probably does indicate sulfate containing compounds (37) in connective tissue probably largely chondroitin sulfate. The viscous material demonstrated by Bensley (2) in regenerating and young tissue and by Maximow (45) in tissue culture, possibly indicates hyaluronate. The rôle of calcium and ascorbic acid in the cement substances of plants and animals has been discussed by Reid (74A).

Robb Smith (75) has studied histologically in vitro the effect of testicular and streptococcal extracts on the muscle of guinea pigs. Reticulin or collagen

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were reported not to be affected, but the reticulin membranes became separated from the muscle fibres. The author suggests that there may be a layer of mucopolysaccharide between the reticulin membrane and the sarcolemma. The changes were absent in heated extracts and were prevented by streptococcus antisera when streptococcal hyaluronidase was employed.

A tentative picture of the development of cement substances in mesodermal tissue combining the admittedly scanty data may be presented as follows: The young fibroblast secretes into the surrounding tissue spaces hyaluronic acid, a precursor of collagen, and a chondroitin sulfate. By local acidification in the immediate neighborhood of the cells the first fibres are produced by the polysaccharides from the native soluble collagen, which denature into the insoluble fibre, on the surface of which lies a sheet of the polysaccharides. With ageing of the fibres, the polysaccharide layer becomes thinner and the hyaluronate is replaced more and more by chondroitin sulfate. Only in metabolically very active connective tissue like that of skin, hyaluronic acid production continues in appreciable quantities. The rôle of ascorbic acid (87) in the process of fibre formation may be that it actually is a component of chondroitin sulfate, replacing in the chain some of the glucuronic acid molecules.

D. *Hyaluronic Acid and Erythrocyte Sedimentation Rate.* Hyaluronic acid in common with other asymmetrical molecules increases the erythocyte sedimentation rate in vitro or after intravenous injections (61). It was further observed in this laboratory that purified testicular hyaluronidase decreased in vitro the sedimentation rate of blood of patients, especially in rheumatic fever. This action was first thought to be due to the hyaluronidase contained in the enzyme preparations. However, it became apparent that hyaluronidase activity as determined by chemical or physico-chemical methods was not proportional to the action on sedimentation rate. This became still more obvious when it was found that enzyme preparations fractionated by lead acetate no longer acted on sedimentation rate, when lead was removed by Na_2S instead of dialysis, while the hyaluronidase activity was not altered. In further experiments by Dr. C. Ragan (unpublished) the mechanism of the action on erythrocytes was found to be due to spherocyte formation, caused by an enzymatic action of unknown nature on the erythrocyte membrane.

The effect of hyaluronate on erythrocytes seems comparable to the precipitation of large particles, such as hemocyanine, liver particles and a number of viruses by some colloids of high molecular weight (10). The precipitation occurs at neutrality and apparently is not due to polar forces. Depolymerized hyaluronate had no effect. It seems remarkable that hyaluronate of high polymerization had a much larger effect quantitatively than either chondroitin sulfate or heparin (see fig. 1 of Cohen and personal communication). The mechanism of this action does not seem clear, but may be connected with complex coacervation (7) and obviously deserves further study.

E. *Hyaluronidase in the Animal Body.* The rôle of testicular hyaluronidase in testis probably is confined to the dispersion of the cumulus cells in the process

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of fertilization (53) (80) (15A). Apparently only mature spermatozoa contain a high concentration of the enzyme. Testes of birds, amphibia and reptiles contain little (12). The mucus of the cervical plug is not acted upon by hyaluronidase (53) (80). The hyaluronidase of human semen has been investigated by several workers (32A) (35A) (86A). Kurzrok et al. by a modified M.C.P. test found a critical value for hyaluronidase of 50 million sperm per cc. Hyaluronidase activity did not run parallel to sperm population. Some apparently normal semen was found to be deficient in hyaluronidase, concomitant with inability to cause fertilization. Mixing of semen and testicular hyaluronidase or application of hyaluronidase in powder form to the cervix was said to have increased considerably the number of successful fertilizations.

No conclusive data are available for the occurrence of hyaluronidase in blood. Intravenously injected enzyme is rapidly eliminated from the circulation.

The demonstration of hyaluronidase in aqueous humor has already been mentioned.

A relatively large concentration of hyaluronidase occurs in skin (57). In press juice of ground rabbit skin very little activity can be demonstrated, but when the material is autolysed in presence of toluol in phosphate buffer of pH 5.0, a relatively large concentration is found in the supernatant solution. Chain and Duthie (9) were unable to find any hyaluronidase in skin. However, the authors likewise failed to obtain a spreading reaction with skin extracts, in contrast to Duran-Reynals' (12) and our experiments. The higher concentration in autolysed material may be due either to the liberation of enzyme bound to cell structures or conversion of inactive into active material. The latter hypothesis seems more likely. Of interest in this connection is the observation of Duran-Reynals that washed glandular tissues of poisonous snakes are almost free of hyaluronidase, whereas it appears with the glandular secretion.

The concentration of potential hyaluronidase in skin may be still higher than that found, since during autolysis considerable destruction of added enzyme occurs.

The occurrence of hyaluronic acid and hyaluronidase in skin may point to a rather rapid turnover of the former. This turnover may be regulated by certain hormones. Luria and Zappasodi (38) found a significant increase in the spreading effect in rabbits treated with luteinizing hormone. Other workers (84) have found a decreased reaction under the influence of follicular hormone. The edema fluid of the sex skin of monkeys in the estrus phase has been reported to be similar to synovial fluid (9). The turgescence of the sex skin of the baboon during the menstrual cycles was studied by Clarke (9A). A considerable part of the water uptake of the skin was apparently bound water since no free edema fluid could be demonstrated. The deturgescence at the end of the estrus cycle supplied the animal with fluid for 11 days. We may assume that this fluid was bound by hyaluronic acid.

Localized hyaluronidase action has been suggested as a possible explanation

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for the bullae formation in pemphigus (38A). However, after intradermal injection of hyaluronidase into normal people and into patients suffering from a variety of rheumatic diseases, bullae were never observed.

F. *Clinical Application.* The use of hyaluronidase in artificial insemination has been mentioned above.

On injection of purified testicular hyaluronidase into the knee joints of patients suffering from rheumatoid arthritis the viscosity of the fluid was remarkably reduced. No change was observed in the underlying disease. One week after the injection, the viscosity of the fluid had returned to the original level (73).

In one patient having a mesenthelioma of the pleura and peritoneum purified testicular hyaluronidase of bull or ram was injected intraperitoneally to facilitate removal of a fluid of honey-like consistency (85). Without hyaluronidase injection, evacuation of the fluid was difficult and incomplete. After injection of 16,000 to 80,000 units of hyaluronidase, fluid of low viscosity could be completely removed in a short time. No immediate harmful effects of the injections were apparent. Injections and paracentesis were repeated at various intervals. The tumor finally did not seem to produce as much fluid as originally. On autopsy large tumor masses were found in peritoneum and pleura with little fluid mostly encapsulated.

A connection between hyaluronic acid and hyaluronidase and rheumatic diseases, especially rheumatic fever, has probably been suspected by many workers. In a number of articles Guerra (16) recently reported the inhibiting effect of sodium salicylate on the spreading of India ink, in rabbits injected with crude testicular hyaluronidase. The area of spreading without hyaluronidase was reduced by 20 per cent with 0.07 and by 31 per cent with 0.10 gram per kgm. of sodium salicylate. With hyaluronidase injection the area of spreading was reduced 57 per cent with 0.07 gram per kgm. and 66 per cent with 0.1 gram per kgm. of sodium salicylate. Sulfadiazine did not decrease the spreading effect of hyaluronidase.

In human subjects intradermal injection of the enzyme with the dye T1824 was said (17) to cause, in individuals with active or inactive rheumatic fever, unique reactions with enormous diffusion of the dye and local edema that sometimes involved the entire arm. Salicylate inhibited the spreading reaction in those cases. The type of reaction was also observed in one male with exanthematic typhus.

In this laboratory salicylate in vitro in equivalent or higher concentration has been found to be without effect on the depolymerization or hydrolysis of hyaluronate. However the marked depressing action on skin diffusion in rabbits has been confirmed (J. A. Coss, personal communication). The explanation of this effect as well as the beneficial action of salicylate on some rheumatic manifestations may be found in an inhibition of hyaluronate production of mesenchymal cells.

CONCLUSIONS

It is obvious from this review that the relationship between bacterial infection and the hyaluronidase system has continued to receive more of the attention

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of investigators than has the metabolism of hyaluronic acid and its rôle in animal physiology and pathology. From the scanty data available, it seems obvious that the functions of the skin, of the ocular fluids, of synovial fluid and of the connective tissues in general must depend in part on the quantity and degree of aggregation of hyaluronic acid. Gels formed by the acid serve partly as the cement which holds cells together. In other structures as in the joint they protect internal surfaces, or they are part of the viscous barriers as in some connective tissues which regulate the exchange of metabolites and water. Thus the physiological aspects of hyaluronic acid as well as of other mesodermal cement substances seem to be of even greater importance than their rôle in infection.

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