HYALURONIDASES OF BACTERIAL AND ANIMAL ORIGIN*

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The system hyaluronic acid-hyaluronidase is of interest for three reasons: (*a*) it may regulate the water metabolism of the ocular and joint fluids, (*b*) it may be one of the factors which determine invasiveness of hemolytic streptococci as well as some of the phenomena connected with hemolytic streptococcus infection, and (*c*) it is intimately connected with the "spreading factor" and consequently with the reactions which may be associated with this factor as, for example, capillary permeability.

The enzyme hyaluronidase was first found in our laboratory in autolysates of a rough Type II pneumococcus (1). Similar enzymes were subsequently demonstrated in ciliary body and iris (2), in a strain of group A hemolytic streptococcus, in *Clostridium welchii*, and in spleen (3). Its occurrence in testis extracts containing the "spreading factor" was first demonstrated by Chain and Duthie (4). Their observations were subsequently confirmed and extended in this laboratory (5).

The relationship between "spreading factor" and hyaluronidase has been discussed in preceding publications (6, 7). It was shown that all fractions containing a hyaluronidase act as "spreading factors." However, in a number of extracts, especially those prepared from streptococcus, no hyaluronidases could be demonstrated, and yet the "spreading" power of these extracts was as marked as that of known hyaluronidases.

In this paper the occurrence, preparation, and properties of hyaluronidases from different sources will be described. Hyaluronidases may be demonstrated by two reactions. They decrease the viscosity of fluids containing hyaluronic acid in a very rapid reaction, which will be called a depolymerization, and secondly in a slower reaction, they hydrolyze hyaluronic acid with the liberation of reducing sugars. The relationship of these two reactions will be discussed.

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Methods

The hydrolysis of hyaluronic acid was measured by the increase in reducing sugar, using as a rule the method of Miller and Van Slyke (8), at the pH optimum of the different enzyme preparations. Equal parts of a 1 per cent solution of hyaluronic acid, to which the calculated amount of sodium hydroxide had been added, and enzyme in M/15 phosphate buffer were mixed, 2 drops of toluene added, and the mixture incubated at 37°C. for the time indicated in the tables. The small reducing values obtained with control enzyme and substrate solutions were subtracted. The per cent hydrolysis was calculated from the glucose equivalents of glucuronic acid and acetyl-glucosamine calculated from the glucosamine concentration previously determined by analysis. The hyaluronic acid was prepared from acetone-dried human umbilical cord by a combination of the various methods described by this laboratory (9). The purity of the samples used was proved by analysis.

It would obviously be advantageous to measure hydrolysis by the liberation of acetyl-glucosamine using the method of Morgan and Elson (10). This was attempted previously in our laboratory without success (9). However Chain and Duthie (4) used this method and it has been used in other investigations so that a description of experiments of this type seems warranted.

Acetyl-glucosamine prepared according to Zuckerkandl (11) was used as a standard. Good recovery could be obtained on adding acetyl-glucosamine to enzyme or to glucuronic acid, with or without incubation, or to hyaluronic acid without incubation with enzyme. If, however, hyaluronic acid was incubated with pneumococcus enzyme and samples withdrawn for acetyl-glucosamine determination at different intervals, the apparent acetyl-glucosamine increased with time to values more than six times the total weights of polysaccharide and enzyme combined. The only explanation which we can offer is that the acetyl-glucosamine liberated by the enzyme has such a structure that it gives a higher condensation and subsequently higher color intensity than the synthetic compound. Obviously this method could not be used to follow the course of the hydrolysis.

Viscosities were determined with an Ostwald viscosimeter in a constant temperature water bath at 37°C. As substrate in the viscosity experiments the sterile pleural fluid of a patient with a mesothelioma was used. It had previously been demonstrated that the viscosity of this material was due to its hyaluronic acid content (12). The fluid was centrifuged at 4000 R.P.M. in an angle centrifuge and kept sterile at 0°C. by the addition of a few drops of chloroform. For the viscosimetric experiments, 5 cc. of a mixture of equal parts of pleural fluid and the substance to be tested were pipetted into the viscosimeter. All viscosities were calculated relative to the viscosity of 0.9 per cent sodium chloride at 37°C.

RESULTS

Pneumococcus Enzyme.—The only strain of pneumococcus in which the enzyme was reported formerly was a rough Type II strain.[1] The enzyme

[1] In this communication the term "rough" as applied to pneumococcus indicates the unencapsulated, non-type-specific variant phase, while "smooth" indicates the encapsulated, type-specific variant phase (13).

now has also been prepared from the smooth form of the same strain, from a Type I strain in the smooth and rough phases, and from a smooth Type VI strain. By the viscosimetry method the enzyme was also demonstrated in one strain of Type III and in another strain of Type VI. It therefore seems probable that the enzyme occurs in all strains of pneumococci.

For the preparation of the enzyme the method previously described (3) was used. Table I gives a summary of the activity of some of these preparations.

The enzyme is contained not only in the organisms but in the medium as well, contrary to our statement in an earlier paper. By using 5 cc. of the supernatant broth, freed of organisms by centrifuging, with 5 cc. of the

TABLE I

Hydrolysis of Hyaluronic Acid by Pneumococcus Enzymes

Source	Enzyme concentration				Enzyme concentration			
	0.50%	0.25%	0.05%	0.01%	0.50%	0.25%	0.05%	0.01%
	per cent hydrolysis (2 hrs.)				*per cent hydrolysis (20 hrs.)*			
Type I smooth	55	55	28	9	59	59	59	52
Type I rough		58	26	8		61	60	55
Type VI	85	75	75	34	92	83	80	77
Type II D/39/R			68	22			92	80
" " "		73	51	22		78	62	61
" " "		78	44	13		88	80	75
" " "		97	64	25		97	92	78

pleural fluid, a marked drop in viscosity was obtained as illustrated in Fig. 1. In this figure there is also plotted an experiment comparing unautolyzed broth culture with the same material after 48 hours autolysis. It is evident that the autolysis damages the depolymerizing enzyme considerably.

Previously we found that the hyaluronidase of pneumococcus was inactivated by iodine and reactivated by arsenite and sulfite (1). Since it has been reported (14) that an enzyme from *Cl. welchii* was inactivated irreversibly by iodine, we repeated the experiment with the pneumococcus hyaluronidase. If the concentration of iodine was too great, irreversible inactivation took place, as is known to happen with other enzyme systems. If, however, a concentration of iodine was chosen so that incomplete inactivation took place, a good deal of the activity could be restored by arsenite, as shown in Table II. High concentrations of reducing agents (arsenite, sulfite) likewise inactivated the enzyme.

Streptococcus Enzyme.—Continued work on the preparation of enzyme

from strain H44, reported in a previous paper (3), has shown an enormous variation in potency of such preparations, many fractions being devoid of activity. This variation is not entirely due to the method of preparation, since the same variation has been encountered when the broth cultures themselves were tested directly by the viscosimetric method. Furthermore the same variation in potency was observed in another strain of group A hemolytic streptococci, and more especially in a group C strain. By the

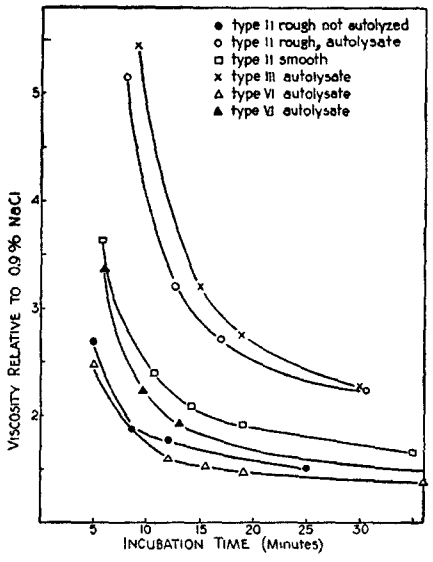

FIG. 1

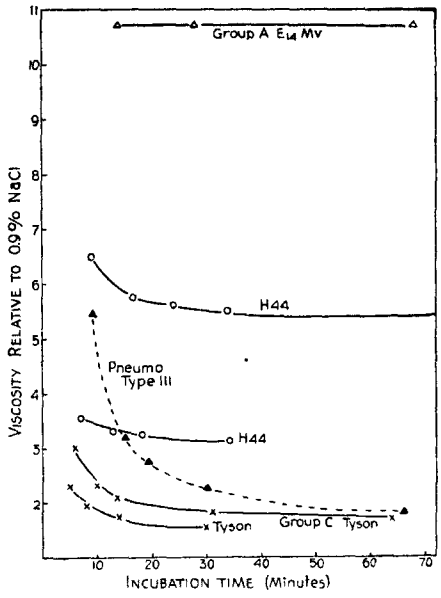

FIG. 2

FIG. 1. Effect of pneumococcal broth cultures on viscosity. The relative viscosity with a saline control was 20.0.

FIG. 2. Effect of streptococcal broth cultures on viscosity. The relative viscosity with a saline control was 20.0.

viscosimetric method some cultures were negative, while others, grown under apparently similar conditions, were among the most potent yet prepared. It was thought that oxidation might possibly play a rôle, but cultures grown anaerobically were found inactive. So far no explanation has been found for the variation in activity.

The hyaluronidase preparations from strain H44 were never as potent as those prepared from pneumococci. It seemed best to extract the streptococci immediately after grinding, since the enzyme deteriorated rapidly when not frozen and dried *in vacuo*. For extraction the use of sodium chloride solutions containing potassium cyanide (0.1 per cent) seemed

advantageous. From the medium after removal of the cells, enzyme preparations could likewise be obtained, though the purification of this material proved quite difficult. The concentration of the enzyme in cultures of H44 seems to have an optimum at about 16 hours of incubation.

TABLE II

Inactivation and Reactivation of the Pneumococcus Enzyme

	Glucose	Per cent of control
	mg.	
Hyaluronic acid + enzyme + NaCl	0.770	100
" " + " + iodine (N/1000)	0.160	21
" " + " + " " + As_2O_3 (N/250)	0.480	62
" " + " + iodine (N/2000)	0.572	74
" " + " + " " + As_2O_3 (N/500)	0.682	89

0.5 cc. samples of enzyme (0.02 per cent in M/15 phosphate buffer pH 5.8) were treated with 0.1 cc. iodine, N/100 and N/200 respectively. After 30 minutes 0.1 cc. arsenious oxide, N/25 and N/50, and 0.2 cc. hyaluronic acid (2.5 per cent) were added. Saline was added to the controls. The mixture was incubated at 37°C. in the presence of toluene for 19 hours, and reducing values determined by the Folin-Wu method. (With iodine and arsenious oxide the ceric sulfate method gave irregular results.)

TABLE III

Hydrolysis of Hyaluronic Acid by Streptococcus Enzymes (H44)

Preparation	Enzyme concentration						Enzyme concentration					
	1.00%	0.50%	0.25%	0.10%	0.05%	0.01%	1.00%	0.50%	0.25%	0.10%	0.05%	0.01%
	per cent hydrolysis (2 hrs.)						*per cent hydrolysis (20 hrs.)*					
1 *a* (16 hr. supernatant)	41	27		7			67	68		40		
1 *b* (16 hr. cells + KCN)			50		14	3			79		59	15
2 *a* (16 hr. cells)		18	13		3	0		60	61		21	4
2 *b* (16 hr. cells + KCN)		27	18		6	0		78	79		24	5
3 *a* (6 hr. supernatant)	2	3		2			10	13		9		
3 *b* (6 hr. cells)		6	4		2	0		39	29		7	2
4 (24 hr. supernatant)	25	15		4			62	67		25		

On storing the activity of the preparations was lost much more rapidly than in the case of pneumococcus preparations. Experiments to restore activity of dried preparations and of broth of low potency were unsuccessful. As reactivating agents sulfite, arsenite, cyanide, cysteine, and ascorbic acid were tried. In Table III the activity of some of the preparations of H44 is reported.

Many attempts were made to demonstrate enzyme in strains of group A hemolytic streptococci other than strain H44. Broth and cells of mucoid, matt, and rough strains were used. No hyaluronidase activity was found in thirteen of the fourteen other strains tested. One group A strain, E_{14} Mv (Dochez), originally obtained from a scarlet fever case, gave a

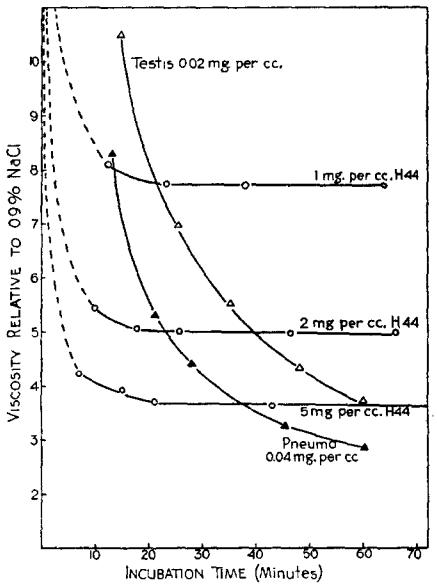

FIG. 3

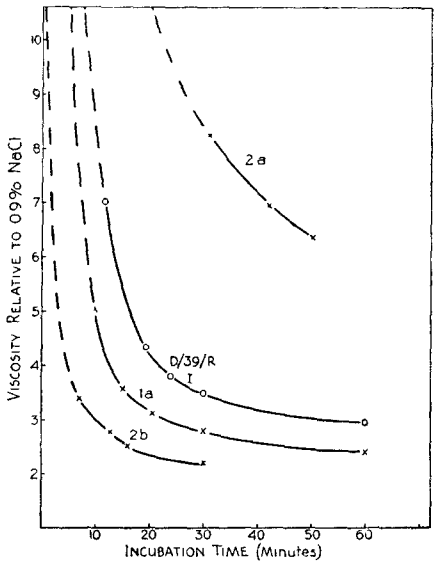

FIG. 4

FIG. 3. Comparison of effects of streptococcus, pneumococcus, and testis enzymes on viscosity. Solutions of dried preparations were prepared in the concentrations indicated in the graph. A mixture of equal parts enzyme solution and pleural fluid was used. The relative viscosity with a saline control was 16.0.

FIG. 4. Effect of leech preparations on viscosity. 0.1 cc. of enzyme solutions containing 1.0 mg. was mixed with 5 cc. pleural fluid. The relative viscosity with a saline control was 178.5. (In this experiment the pleural fluid was used undiluted. Note the high original viscosity.)

positive depolymerase action in one out of three samples. Of two group C strains tested, one was found inactive, and one highly active, though extremely variable. In two strains of *Streptococcus viridans* no enzyme activity was demonstrated. In the light of the variation encountered in streptococcal preparations, further study may reveal enzyme in some of the negative cases, especially since most of these were tested only once.

In Fig. 2. the drop in viscosity produced by broth cultures of various strains of streptococci is illustrated. It seems remarkable that with the

group A strains no further decrease in viscosity occurs after the initial drop. In the case of a Type III pneumococcus preparation, which is included for comparison, the drop in viscosity is continuous.

The active flavianates prepared from the ground organisms give the same flat curves as those of the broth of the supernatant cultures. In Fig. 3, the viscosity measurements with a flavianate prepared from strain H44 are given. For comparison a testis and a pneumococcus preparation are included in this figure. It is evident that the activity of the streptococcal enzyme preparations stops after the initial action.

Leech Enzyme.—Claude has found that extracts of leech heads contain "spreading factor" (15). He further found that such extracts decrease the viscosity of chicken tumor extracts (16).

TABLE IV

Hydrolysis of Hyaluronic Acid by Leech Enzyme Preparations

	Enzyme concentration			Enzyme concentration		
	0.25%	0.05%	0.01%	0.25%	0.05%	0.01%
	per cent hydrolysis (2 hrs.)			*per cent hydrolysis (20 hrs.)*		
1 *a*	37	26	13	67	47	24
1 *b*		10	6		26	15
2 *a*	8	6	2	23	25	14
2 *b*	36	29	23	37	36	36
Pneumococcus D/39/R I	51	23	7	64	55	40

In Table IV the hyaluronidase activity of some preparations from leech heads is recorded. Preparation 1 *a* was made by freezing and drying a solution purified by the copper sulfate method of Dr. Claude.[2] Preparation 1 *b* was made from extract 1 *a* by repeating the copper sulfate precipitation and precipitating the enzyme by sodium flavianate. Considerable activity was lost by this procedure. Preparations 2 *a* and 2 *b* were made from an aqueous extract of leech heads filtered through a Berkefeld filter. A large part of the protein was removed by acidification with a few drops of normal sulfuric acid and fractional precipitation with sodium flavianate. Preparation 2 *a* is the first fraction obtained by carefully adjusting the pH and preparation 2 *b* is the second fraction obtained after further acidification. It is strange that preparation 2 *b* gave the same hydrolysis after 20 hours, regardless of the amount of enzyme present. The leech preparations had a lower activity than our usual pneumococcus preparations. The pneumococcus preparation is included in the table since this sample had been used in the experiment shown in Fig. 4. The kinetics of the preparations from leech and pneumococcus are certainly different and a comparison between the two seems impossible. The hydrolysis was carried out at pH 5.4 which was found to be the optimal pH.

[2] We thank Dr. Albert Claude for this material.

In Fig. 4 the depolymerizing action of the leech preparations is shown. For comparison a pneumococcus preparation was run simultaneously. This figure shows that the same concentrations of two leech preparations have a more pronounced action on viscosity than the pneumococcus preparation used in this experiment. This particular pneumococcus preparation proved to be one of the least potent from this source.

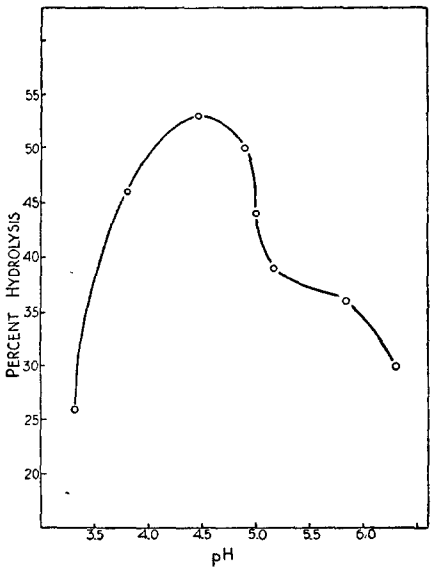

FIG. 5

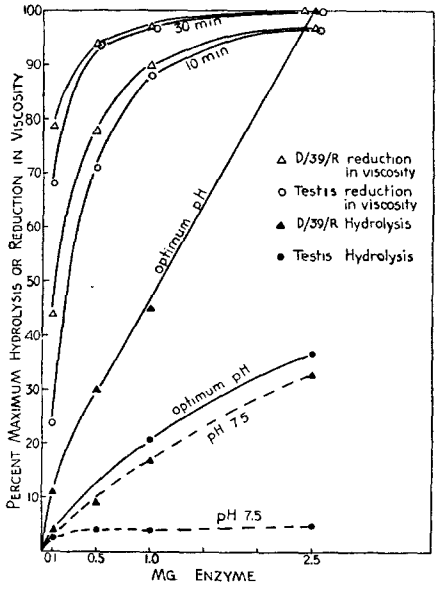

FIG. 6

FIG. 5. pH activity curve of the testis enzyme. Preparation 1*b* of Table V and hyaluronic acid in concentrations of 0.5 per cent were mixed at varying pH values using 0.3 M citrate buffers. After 21 hours incubation at 37°C., the pH was determined with a glass electrode.

FIG. 6. Hyaluronidase and depolymerase activities of pneumococcus and testis enzymes.

Testis Enzyme.—Testis enzymes were prepared by the method of Morgan and McClean (17), or by ammonium sulfate fractionation, or by a combination of the two with acetone or flavianate precipitation.

In Fig. 5 the pH curve of one enzyme preparation (No. 1*b* of Table V) is given. It can be seen from this figure that the optimal activity is found at pH 4.4, while the pH optimum of the pneumococcus enzyme was at pH 5.6 to 5.8. Furthermore the pH curve of the testis enzyme shows a break at about pH 5.8. This break, as well as the optimum of the curve, have been reproduced in other experiments.

In Table V the activity of a number of different preparations, tested at pH 4.4 is recorded.

It is evident that the hydrolysis in 20 hours rarely exceeds 50 per cent. If the hydrolysis is prolonged to about 60 hours the hydrolysis reaches approximately 65 per cent. A more complete hydrolysis was not achieved. However this is not due to enzyme destruction. This was demonstrated in the following experiment. Testis enzyme was incubated with hyaluronic

TABLE V

*Hydrolysis of Hyaluronic Acid by Testis Enzymes**

	Enzyme concentration				Enzyme concentration			
	1.00%	0.50%	0.25%	0.05%	1.00%	0.50%	0.25%	0.05%
	per cent hydrolysis (2 hrs.)				*per cent hydrolysis (20 hrs.)*			
1 *a*		33	29	16		40	35	29
1 *b*		42	30	16		47	42	34
2	23	20			52	38		
3	35		18		52		34	
4		37	33	16		43	41	23
5			19	5			28	13

1 *a*. Flavianate prepared from testis extract purified according to Morgan and McClean.

1 *b*. Enzyme solution, according to Morgan and McClean, frozen and dried.

2 and 3. N/10 acetic acid extract precipitated with ice cold acetone.

4. N/10 acetic acid extract fractionated with ammonium sulfate. Fraction between 50 per cent and saturation dialyzed and solution frozen and dried.

5. Supernatant solution after 50 per cent ammonium sulfate precipitation fractionated by acidification as flavianate. Third fraction precipitated at pH 3.5 after removal of precipitates at pH 5 and 4.

* 0.3 M citrate buffer, pH 4.4 was used in these experiments.

acid for 42 hours. Samples were taken at 2, 20, and 42 hours. The solution was then divided into four fractions as shown in Table VI.

Table VI demonstrates that the rate of hydrolysis by the testis enzyme falls off rapidly. Dilution with saline leads to an increased hydrolysis. The addition of new testis enzyme does not alter this rate appreciably, indicating inhibition by the split products. On the other hand addition of new substrate causes the resumption of hydrolysis at almost the initial rate (19 and 41 per cent hydrolysis as against 21 and 48 per cent). Addition of pneumococcus enzyme causes almost complete hydrolysis of the residual substrate left from the action of the testis enzyme.

The data of this experiment as well as the pH curve are best explained by the assumption that there are two enzymes in these preparations, the main fraction hydrolyzing the long chain molecules into aldobionic acid

units and the other enzyme present in smaller concentration hydrolyzing the aldobionic acid units into monosaccharides. The hypothetical dual nature of this system is likewise the best explanation of its influence on viscosity.

The depolymerizing action of the testis as well as the leech enzyme is considerably greater than that of the pneumococcus enzyme when compared on the basis of their respective hydrolyzing activities on hyaluronic acid. In the following experiment different concentrations of testicular and pneumococcal enzymes were incubated at their respective pH optima and at pH 7.5 (the pH at which the change in viscosity was determined)

TABLE VI

Effect of Substrate and Enzyme Concentration on the Kinetics of the Testis Enzyme

	2 hrs.		20 hrs.		42 hrs.
	Total glucose	Hydrolysis	Total glucose	Hydrolysis	Hydrolysis
	mg.	*per cent*	*mg.*	*per cent*	*per cent*
I. Hyaluronic acid (5 mg. per cc.) + testis enzyme (10 mg. per cc.)		21		48	55
(*a*) 1 cc. I (after 42 hrs.) + 0.5 cc. saline	2.05	57	2.31	65	
(*b*) " " " " " " + 0.5 cc. testis enzyme (10 mg.)	2.16	60	2.48	70	
(*c*) " " " " " " + 0.5 cc. pneumococcus enzyme (0.2 mg.)	2.87	81	3.08	87	
(*d*) 1 cc. I (after 42 hrs.) + 0.5 cc. hyaluronic acid (5 mg.)	2.70		3.75		
Hydrolysis of added polysaccharide (*d* minus *a*)	0.65	19	1.44	41	

with hyaluronic acid. The reduction was measured after 2 and 20 hours incubation. The same amounts of enzyme dissolved in 5 cc. of 0.9 per cent sodium chloride were mixed at 37°C. with 5 cc. of pleural fluid and the viscosity determined at different intervals.

Fig. 6 clearly demonstrates that the hydrolysis by the testis enzyme at the optimal pH or at the alkaline pH is only a fraction of that by the pneumococcus enzyme, while the depolymerizing actions of the two are identical with the exception of the lowest concentrations. If the depolymerizing action of the two enzymes was calculated on the basis of their respective hyaluronidase activities at pH 7.5, the pneumococcus enzyme ought to have about the same activity in 0.1 mg. as the testis enzyme in 2.5 mg. Some preparations have shown even greater discrepancy.

Spleen Enzyme.—Spleen extracts were tested for hydrolysis of β-glucuronides and of hyaluronic acid, in order to determine whether the two sub-

strates were attacked by the same enzyme. Extracts were prepared from beef spleen according to Fishman (18). Some of the extracts were precipitated as flavianates.

One preparation failed to hydrolyze hyaluronic acid, though it gave good hydrolysis of borneol- and pregnandiol-β-glucuronide. Another fraction hydrolyzed both hyaluronic acid and β-glucuronides. On the other hand neither pneumococcus, testis, nor leech extracts hydrolyzed the two β-glucuronides. Thus hyaluronidase does not seem identical with β-glucuronidase.

Skin Enzyme.—The occurrence of "spreading factor" in rabbit skin was reported by Claude and Duran-Reynals (19). They found their skin

TABLE VII

Activity of the Spleen Enzyme

Enzyme preparation	Hydrolysis of hyaluronic acid			Hydrolysis of pregnandiol glucuronide		
	2 hrs.	20 hrs.	44 hrs.	2 hrs.	20 hrs.	44 hrs.
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
1	0	0		15	46	
2	3	19	26	36	70	67

The pregnandiol glucuronide was a sodium salt obtained from Dr. Hans Hirschmann, Philadelphia.

extracts greatly inferior to those prepared from testis in regard to "spreading" action.

In our experiments the trunk of a rabbit was shaved, and the hair further removed from the skin with barium sulfide paste. After washing off the barium sulfide, an area of about 25 by 30 cm. weighing about 80 gm. was dissected and ground in the Latapie mill with M/15 phosphate buffer of pH 5.0. The suspension was autolyzed at 37°C. for 16 hours in the presence of toluene and centrifuged. The residue was extracted once with 50 cc. of 0.9 per cent sodium chloride. The total fluid was 135 cc. On mixing 5 cc. of this extract with 5 cc. of pleural fluid, the relative viscosity dropped from 19.0 to 8.9 in 169 minutes. 116 cc. of the skin extract were adjusted to pH 6.5 and precipitated with lead acetate as in the testis experiments. The supernatant solution after dialysis was frozen and dried. 24 mg. were obtained. This material gave the hydrolysis of hyaluronic acid shown in Table VIII.

It is obvious that the amount of enzyme in the rabbit skin is considerable, especially in view of the probable incomplete extraction and the inactivation of enzyme by autolysis. Extraction at pH 7.0 without autolysis gave an enzyme preparation of only slight activity. In cornea, a tissue closely related to skin, the enzyme has likewise been demonstrated (20).

Substrates Other than Hyaluronic Acid.—In a previous paper (3) a list of substrates was given for enzyme preparations from *Cl. welchii* and pneumococcus. The pneumococcus enzyme acted only on hyaluronic acid and on a trisulfuric acid ester prepared from it, while the *welchii* enzyme hydrolyzed other substrates.

Meanwhile we have found two other natural substrates of our preparations from pneumococcus. One is the mucoitinsulfuric acid of cornea (5, 20), which is apparently a monosulfuric acid ester of hyaluronic acid. This cornea polysaccharide is isomeric with the acid polysaccharide of pig gastric mucosa (21, 22). The gastric mucosa polysaccharide has a rotation quite different from the cornea polysaccharide and it is completely resistant to the pneumococcus enzyme. It is, however, hydrolyzed by the *Cl. welchii* enzyme.

TABLE VIII

*Hydrolysis of Hyaluronic Acid by Skin Enzyme**

Enzyme concentration	Hydrolysis		
	2 hrs.	20 hrs.	44 hrs.
per cent	*per cent*	*per cent*	*per cent*
1.00	27	32	35
0.50	13	19	22
0.25	8	12	15
0.10	4	6	9
0.05	3	5	9
0.01	1	2	3

* In this experiment, M/15 phosphate buffer, pH 5.8 was used. Whether this is the optimal pH has not yet been determined.

The other substrate of our pneumococcal preparations was isolated from submaxillary gland. This substance contains acetyl-glucosamine and gluconic acid in equimolar concentrations. The hydrolysis of this substance as compared to hyaluronic acid is quite slow, and may not be due to the hyaluronidase. The submaxillary gland acid is hydrolyzed by an enzyme contained in beef submaxillary gland and in human saliva. The latter does not attack hyaluronic acid. The submaxillary gland compound and its enzymatic hydrolysis will be described in another paper.

In Table IX a summary is given of a number of substances tested against pneumococcal and testicular enzymes. The latter hydrolyzed chondroitinsulfuric acid from cartilage, as did the *welchii* enzyme,[3] with the liberation

[3] The enzyme from *Cl. welchii* has been used by the Boston workers to identify a "mucin" from connective tissue (14). Probably all connective tissue contains chon-

of sulfuric acid. It also hydrolyzed a sulfuric acid containing carbohydrate prepared from pigskin,[4] which probably is identical with chondroitinsulfuric acid. Preparation 4 of Table V hydrolyzed a pure barium salt of chondroitinsulfuric acid from cartilage in 20 hours to 24 per cent at a pH of 5.8. The concentration of substrate and enzyme was 5 mg. per cc. This hydrolysis of chondroitinsulfuric acid furnishes to our knowledge definite proof for the first time that the animal body possesses enzymes against this important body constituent.

TABLE IX

Hydrolysis of Substrates Other than Hyaluronic Acid

Substrate	Source of enzyme		
	Testis	Leech	Pneumococcus
Mucoitinsulfuric acid (from gastric mucin)	−	−	−
Chondroitinsulfuric acid	+	−	−
Pigskin polysaccharide (sulfur-containing fraction)	+		−
Heparin	−	−	−
Cornea polysaccharide	+		+
Neutral polysaccharide (from gastric mucin)	−	−	−
Acid polysaccharide from pregnancy urine (hyaluronic acid?)		−	+
Borneol glucuronide	−	−	−

Relationship of Hyaluronidase to "Spreading Factor"

The relationship between hyaluronidase and "spreading factor," which was investigated in a previous paper (7), has in the meantime been discussed by a number of other authors (23–26). New evidence for the identity of "spreading factor" and hyaluronidase has been found in the demonstration of "mucinase" in a number of snake venoms, which were known to contain the "spreading factor" (24, 25). It has further been reported that diazobenzenesulfonic acid, azoproteins, and ascorbic acid oxidized with hydrogen perioxide decrease the viscosity of synovial fluid (25, 26). All these agents have been reported as active "spreading factors." In our experience, none of the latter agents has decreased the viscosity of the pleural fluid. The discrepancy between our findings and those reported by others may be explained by the presence of traces of enzyme or inactive enzyme in the synovial fluid and its absence in the pleural fluid.

droitinsulfuric acid. The hydrolysis of the latter as well as of other mucopolysaccharides by extracts from *Cl. welchii* ought to caution against its use for the differentiation of various mucoids.

[4] Two polysaccharides were isolated from skin. The one was sulfate-free and proved to be identical with hyaluronic acid from other sources. The other was not hydrolyzed by pneumococcal hyaluronidase and is probably chondroitinsulfuric acid, though the rotation of some of the preparations was different from that of chondroitinsulfuric acid from cartilage. The experimental data of this work will be published elsewhere.

The only serious discrepancy in the acceptance of the theory that hyaluronidase is identical with "spreading factor" lies in the failure to demonstrate hyaluronidase in the majority of streptococcal preparations which produced "spreading." At the present time one can only say that this failure means that hyaluronidase could not be demonstrated by *in vitro* methods. The great lability of the streptococcal enzyme is apparent, first, from the variation in enzyme content of different batches of the same organism, grown under conditions as identical as possible, and second, from the shape of the viscosity curves of active hyaluronidase preparations. The shape of these curves probably indicates that the enzyme becomes inactivated or destroyed during the experiment. Though no conditions were found to reactivate such extracts or to prevent inactivation *in vitro*, it is conceivable that they may be reactivated *in vivo*, in the spreading test.

On the other hand it is possible that such inactive streptococcal preparations may activate the hyaluronidase preformed in the skin. The latter mechanism seems probable in the case of simple chemical substances having spreading activity. As a rule however, the spreading reactions due to simple chemical substances are weaker or slower than the reactions caused by streptococcal material. The mechanism of "spreading" produced by streptococcal material as well as the rôle played by the enzyme in other activities of hemolytic streptococci require further elaboration.

DISCUSSION

Hyaluronidase preparations have been tested for (*a*) hydrolysis of hyaluronic acid, and (*b*) influence on the viscosity of fluids containing hyaluronic acid. The discrepancy of hyaluronidase and depolymerase activity has been stressed in the comparison between pneumococcal and testicular enzyme preparations. The question arises whether the two reactions are due to two different enzymes.

At slightly alkaline pH the testicular enzyme has only a negligible hydrolyzing activity, while at the same pH pneumococcus enzyme has still about 50 per cent of the activity at the pH optimum. Yet at an alkaline pH, the testicular enzyme is equal or in many instances superior to the pneumococcus enzyme in catalyzing depolymerization. Furthermore in the presence of protein in excess over hyaluronic acid, as in the pleural fluid, the hydrolysis of hyaluronic acid is greatly depressed (3) so that it seems doubtful that any opening of glucosidic linkages occurs during the short time in which the depolymerization may be achieved.

On the other hand the depolymerase action is a specific enzymatic effect. Substances having no hyaluronidase activity in our experience failed to influence the viscosity of the pleural fluid at constant pH and salt concentrations. When hyaluronidase action was demonstrable in a material, depolymerase action likewise was apparent. Furthermore, when the hyaluronidase activity of a preparation was diminished or destroyed by heating or by poisons, the depolymerizing action was likewise diminished or destroyed.

It was previously pointed out that the pleural fluid is a gel (12). Hyaluronic acid isolated from it was shown to have a considerable viscosity, which however accounted for only a fraction of the viscosity of the native fluid. In the Tiselius apparatus at a pH of 7.8, the mobility of the isolated hyaluronic acid was the same as the mobility of the hyaluronic acid in the native fluid. It was concluded from this that hyaluronic acid occurred in the fluid either free or as an easily dissociated protein salt. The protein-salt formation itself probably does not account for the abnormal viscosity, since at the pH of the fluid and in the absence of basic proteins, the dissociation must be quite complete. In any case, hydrolysis of the pleural fluid by proteolytic enzymes (trypsin and papain) altered the viscosity to a negligible degree. Thus the component which is responsible for the structure of the fluid is hyaluronic acid itself and the loss of structure must be due not to hydrolysis but to depolymerization of this substance.

The following hypothesis would explain how the enzyme specific for the hydrolysis might catalyze the depolymerization without any hydrolysis taking place. We assume the hydrolysis to be a reaction involving several steps. The formation of a primary substrate-enzyme complex would be sufficient to disrupt the weak bonds holding the micellae together. The subsequent step reactions necessary for opening of glucosidic linkages however would not take place at the alkaline pH. The primary substrate-enzyme complex could therefore again dissociate and no hydrolysis would take place.

The relationship between depolymerization and hydrolysis as found in the hyaluronidase preparations is probably quite a common enzymatic phenomenon. Similar relations have been encountered in the amylase-starch (27), in the pectinase-pectin (28), and in the lysozyme-sarcinae carbohydrate (29) systems.

CONCLUSIONS AND SUMMARY

Hyaluronidase has been investigated in various strains of pneumococci and hemolytic streptococci, and in some material of animal origin. The enzyme activity was measured by a viscosimetric method using as a substrate a fluid containing hyaluronic acid as the viscous component, and by the hydrolysis of pure hyaluronic acid into its reducing components.

In pneumococci the enzyme was demonstrated in all types and in all strains tested, including smooth and rough forms of Types I, II, III, and VI.

In hemolytic streptococci the enzyme from strain H44, group A, reported previously, was further investigated. In this strain, as well as in

other hemolytic streptococci containing the enzyme, great variability of the enzyme concentration was found. Furthermore, the enzyme proved to be very labile, giving in the viscosimetric measurements a typical stoppage of the activity initially present. In 13 out of 14 other strains of group A organisms investigated, no enzyme was demonstrable, but the variation in activity in the enzyme-active strains renders the negative findings inconclusive. A very active enzyme, though of great variability, was found in one group C strain.

The enzyme was prepared from the leech in confirmation of the work of Claude.

The enzyme from testis showed a maximum at pH 4.4 in contrast to the optimum of 5.8 in pneumococcal, streptococcal, and *Cl. welchii* preparations. The pH curve of the testis enzyme indicated, however, a second optimum coinciding with that of the bacterial enzymes. The hydrolysis further indicated a break at about 50 per cent hydrolysis, indicating primarily the hydrolysis down to aldobionic acid units. The depolymerizing action of testis enzyme is more marked than that of pneumococcal enzyme. The results have been interpreted as due to the presence of two enzymes, one attacking the long chain molecule, the other hydrolyzing the aldobionic acid formed.

The enzyme was further prepared from beef spleen. Here the hydrolysis of β-glucuronides was compared to that of hyaluronic acid. The two actions apparently are catalyzed by two distinct enzymes.

Enzyme preparations were further obtained from rabbit skin. Since hyaluronic acid has also been found in the skin, this organ may play a considerable rôle in the metabolism of hyaluronic acid.

In addition to hyaluronic acid, it has been shown that hyaluronidases also hydrolyze the sulfuric acid containing polysaccharide of the cornea. This polysaccharide has previously been characterized as a natural sulfuric acid ester of hyaluronic acid. The pneumococcal enzyme preparations also attacked a polysaccharide acid prepared from submaxillary gland, which is not hyaluronic acid. However, it is believed that this hydrolysis is due to a second enzyme contained in the preparations. The testis enzyme, on the other hand, attacked chondroitinsulfuric acid and also contained a sulfatase.

The depolymerizing action of hyaluronidase has been discussed. It is concluded that depolymerization and hydrolysis are probably due to the same enzyme attacking hyaluronic acid. It is suggested that the first attack of the enzyme does not cause an opening of glucosidic linkages. The available evidence indicates that the viscosity of the natural fluids is

not due to macromolecules but to micellae formation, and that these micellae are depolymerized by the enzymatic reaction. It is assumed that the depolymerization is due to a primary enzyme-substrate reaction, which in itself is insufficient to open the glucosidic linkages. The latter reaction involves further steps.

The relationship between hyaluronidase and "spreading factor" has been discussed anew. Though more data have been reported pointing to the identity of hyaluronidase and "spreading factor," our inability to demonstrate hyaluronidase in streptococcal material of high "spreading" potency, is still a serious obstacle to the unitarian theory. However, it seems possible that the streptococcal material may contain reversibly inactive enzyme which may be reactivated *in vivo*.

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