THE HYDROLYSIS OF HYALURONIC ACID BY BACTERIAL ENZYMES*†

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In previous papers from this laboratory (1) it has been shown that vitreous humor and umbilical cord contain a mucopolysaccharide which apparently consists of acetyl-glucosamine and glucuronic acid in equimolar concentrations. This mucopolysaccharide has been designated by the term hyaluronic acid. Mucopolysaccharides which appear to be chemically identical with hyaluronic acid have also been isolated from the mucoid phase of group A hemolytic streptococci (2), from synovial fluid (3), and from fowl sarcoma (4). It has further been shown (5) that the autolytic system of pneumococcus contains a factor which hydrolyzes hyaluronic acid.

In the present paper an improved method for the preparation of the hydrolytic enzyme from pneumococci is described. The same enzyme may be prepared from group A hemolytic streptococci, from *Clostridium welchii*, and from beef spleen. Data are also presented to show that the hydrolyzing enzyme is not identical with the bacteriolytic system of the pneumococcus. Finally, brief reference is made to experiments suggesting a possible relation between the carbohydrate-splitting enzyme and the growth phase and virulence of group A hemolytic streptococci.

EXPERIMENTAL

Preparation of the Enzyme from Pneumococci.—The strain of pneumococcus employed in all experiments was a non-encapsulated, non-type-specific variant of a Type II pneumococcus. (According to older terminology the strain was in the rough phase; according to the terminology proposed by Dawson (6) the strain was in the smooth phase.)

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Approximately 9 liters of culture were grown for 5½ hours in beef infusion broth. The organisms were separated by centrifuging, suspended in saline to one-twentieth of the original volume, and allowed to autolyze for 48 hours at 37°C. with an excess of toluene. After adjusting the pH to 4.6–4.8, the suspension was centrifuged in the cold for one hour. The precipitate, which was subsequently found to contain the greater portion of the bacteriolytic system, was purified by resolution and reprecipitation. The supernatant, which was found to contain the bulk of the carbohydrate-splitting enzyme, was further acidified to a pH of about 3.5–4.0 by the addition of normal H_2SO_4 and allowed to stand for an hour in the refrigerator. Additional precipitate was removed by centrifugation. The carbohydrase was precipitated from the supernatant solution by adding one-twentieth of its volume of 4 per cent sodium flavianate.[1] A yellow precipitate usually formed immediately. This precipitate represented the carbohydrase fraction. After centrifugation it was suspended in 50 cc. of water, dissolved by careful addition of normal NaOH, and reprecipitated by a few drops of 10 per cent acetic acid. It was then centrifuged again, suspended in 10 cc. of water, neutralized with NaOH, and frozen and dried *in vacuo*. The yield varied from 50 to 120 mg. It was found that the powder lost little of its activity when kept at room temperature for periods as long as 6 months.

The foregoing method of preparing the enzyme was found to possess certain disadvantages. For example, the enzyme solution could not be readily sterilized and further purification after conversion to the flavianate could not be readily effected. On the other hand, the method as described proved practicable and gave roughly quantitative results.

Hydrolysis of the Polysaccharide.—Hydrolysis of the polysaccharide was carried out as follows: 0.1 mg. of the powder was incubated at 37°C. with 5 mg. of the polysaccharide in the presence of toluene at pH 6.0. Almost immediately loss of viscosity of the solution was observed. Reducing values were determined after 2 hours, 20 hours, and 44 hours. In the first experiments reducing sugar was determined by the Hagedorn-Jensen method (7) omitting the zinc hydroxide precipitation. In the later experiments the ceric sulfate method (8) was used. The theoretical reducing value of a given preparation was calculated from the acetyl-glucosamine and glucuronic acid content as determined by analysis and expressed as equivalents of glucose.

Activity of the Enzyme

In Table I data are presented which demonstrate the activity of the different enzyme preparations in a concentration of 0.01 per cent.

In Table II the hydrolysis of the polysaccharide by different concentrations of one preparation of the enzyme is shown.

In the following experiment the action of the enzyme was tested with the free polysaccharide acid as substrate as compared with the protein salt of the acid.

[1] National Aniline and Chemical Company No. 10 (4) naphthol yellow S, certified food color, can be used without recrystallization. Other samples were found to be impure.

The protein salt used in this experiment was a reprecipitated synovial "mucin," containing by analysis 6.17 per cent uronic acid and 7.55 per cent hexosamine (3). The free polysaccharide acid was also prepared from synovial fluid and was used in a concentration of 0.5 per cent. The concentration of the mucin was 2 per cent to give a polysaccharide concentration similar to that of the free polysaccharide acid.

TABLE I

Hydrolysis of Hyaluronic Acid by Different Pneumococcus Enzyme Preparations

Enzyme concentration 0.01 per cent Preparation No.	Hydrolysis		
	2 hrs.	20 hrs.	44 hrs.
	per cent	*per cent*	*per cent*
1	42	88	95
2	45	85	
3	40	78	87
4	40	89	90

TABLE II

Hydrolysis of Hyaluronic Acid with Varying Concentrations of Pneumococcus Enzyme

Enzyme concentration Preparation 1	Hydrolysis		
	2 hrs.	20 hrs.	44 hrs.
per cent	*per cent*	*per cent*	*per cent*
0.10	89	95	95
0.05	87	94	95
0.025	71	95	96
0.010	42	88	95

TABLE III

Comparison of Hydrolysis of Free Polysaccharide Acid and Protein Salt of the Acid by the Pneumococcus Enzyme

Enzyme concentration 0.025 per cent	Hydrolysis		
	2 hrs.	22 hrs.	45 hrs.
	per cent	*per cent*	*per cent*
Free polysaccharide acid	71	95	96
Protein salt of the acid	36	52	69

The results in Table III indicate that the enzyme acts on the protein salt as well as on the free polysaccharide but only at about half the rate.

Preparation of the Enzyme from Other Sources than Pneumococci

A. Preparation of the Enzyme from Group A Hemolytic Streptococci.—The strain of streptococcus employed, designated as H44, was originally isolated

in this laboratory from a fatal case of septicemia. It is of possible significance that the colonies produced by this strain on neopeptone blood agar plates were consistently matt in character. Mucoid colonies were not observed.

The organisms from 5.5 liters of a 16 hour culture in beef infusion broth were centrifuged, suspended in saline to one-twentieth of the original volume, dried in the frozen state, and ground in a lyophile apparatus for 4 hours. The ground material was extracted with one-fifteenth of the original volume of saline and the pH adjusted to 4.6. The remaining steps in the procedure were the same as those described for the preparation of the enzyme from pneumococcus. 156 mg. of flavianate were obtained.

The hydrolysis produced by this enzyme preparation is shown in Table IV. From the results presented it is seen that the carbohydrase action of

TABLE IV

Hydrolysis of Hyaluronic Acid by the Streptococcus Enzyme

Enzyme concentration 0.25 per cent			Enzyme concentration 0.10 per cent			Enzyme concentration 0.05 per cent		
Hydrolysis			Hydrolysis			Hydrolysis		
2 hrs.	20 hrs.	44 hrs.	2 hrs.	20 hrs.	44 hrs.	2 hrs.	20 hrs.	44 hrs.
per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
25	77	85	11	43	57	6	23	32

the streptococcus enzyme was weaker than that obtained from pneumococcus. In all other respects, however, it exhibited the same characteristics.

B. Preparation of the Enzyme from Clostridium welchii.—The occurrence in an unidentified strain of *Cl. welchii* of an enzyme which diminished the viscosity and gave rise to reducing substances from synovial mucin was reported by Robertson, Ropes, and Bauer (9). This finding suggested the presence of an enzyme in *Cl. welchii* similar to that found in pneumococci and in streptococci. By procedures similar to those already described an enzyme was obtained from the Lister A strain of *Cl. welchii.*

700 to 800 cc. of culture were grown from 24 to 48 hours at 37°C. in beef infusion broth. The organisms were removed by centrifugation. The remaining material was acidified to pH 4.5 and centrifuged. The acid supernatant was treated with sodium flavianate in the manner previously described. Between 70 and 180 mg. of flavianate were obtained. In one experiment half of the broth was salted out with $(NH_4)_2SO_4$ and the other half prepared as above. In another experiment the broth was precipitated by nine volumes of ice cold acetone. The resulting precipitate was washed with acetone and ether and dried *in vacuo*. The loss of enzyme by this treatment, however, was very considerable although the resulting powder still retained some activity.

The properties of the enzyme obtained from the culture of *Cl. welchii* were so similar to those of the enzymes obtained from the other two organisms that the identity of the two seems probable. The results of the hydrolysis of the polysaccharide are shown in Table V and a comparison of the activity of the enzyme with that obtained from pneumococcus is shown in Fig. 1.

Comparison of the activity of the two enzymes shows that it was necessary to use the *Cl. welchii* preparation in concentrations twenty-five times as great as in the case of the pneumococcus preparation. The activities of two preparations were compared at different pH values and with different buffers. The pH was determined by a glass electrode. The time of incubation was 15 hours. Fig. 1 shows that the enzyme preparation from pneumococcus in a concentration of 0.01 per cent effected nearly the same degree of hydrolysis as the *Cl. welchii* preparation in a concentration of 0.25 per cent.

TABLE V

Hydrolysis of Hyaluronic Acid by the Clostridium welchii Enzyme

Preparation No.	Enzyme concentration 0.25 per cent			Enzyme concentration 0.05 per cent			Enzyme concentration 0.01 per cent		
	Hydrolysis			Hydrolysis			Hydrolysis		
	2 hrs.	20 hrs.	44 hrs.	2 hrs.	20 hrs.	44 hrs.	2 hrs.	20 hrs.	44 hrs.
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
1	53	77		5	53		2	6	
2	77	84		13	78		5	33	
3	86	85	91	62	84	90	15	84	87
4*a*	44	74		3	29		1	13	
4*b**	35	73							

* This fraction was an $(NH_4)_2SO_4$ precipitate (55 per cent saturation) dissolved in 25 cc. of water. It was diluted to correspond to an equivalent amount of the flavianate.

Two points of distinction were noted between the *Cl. welchii* and pneumococcus preparations. In the case of the former, the supernatant broth culture proved to be a potent source of enzyme, whereas none could be extracted from the bacterial cells themselves. In the case of pneumococcus the enzyme was found in the bacterial cells.

The second distinction between the two preparations concerned the wider range of activity exhibited by the *Cl. welchii* preparation. The flavianate fraction from *Cl. welchii* contained a number of carbohydrate-splitting enzymes which were not present in the pneumococcal and streptococcal preparations. The preparations made from *Cl. welchii* hydrolyzed starch,

chondroitin-sulfuric acid and mucoitin-sulfuric acid from gastric mucosa (10–12), and, most interesting of all, a neutral polysaccharide isolated from pig gastric mucosa (11). This polysaccharide, which was isolated in our laboratory several years ago, is apparently identical with blood group A substance. The experiments dealing with the hydrolysis of this substance will be discussed in a separate paper.

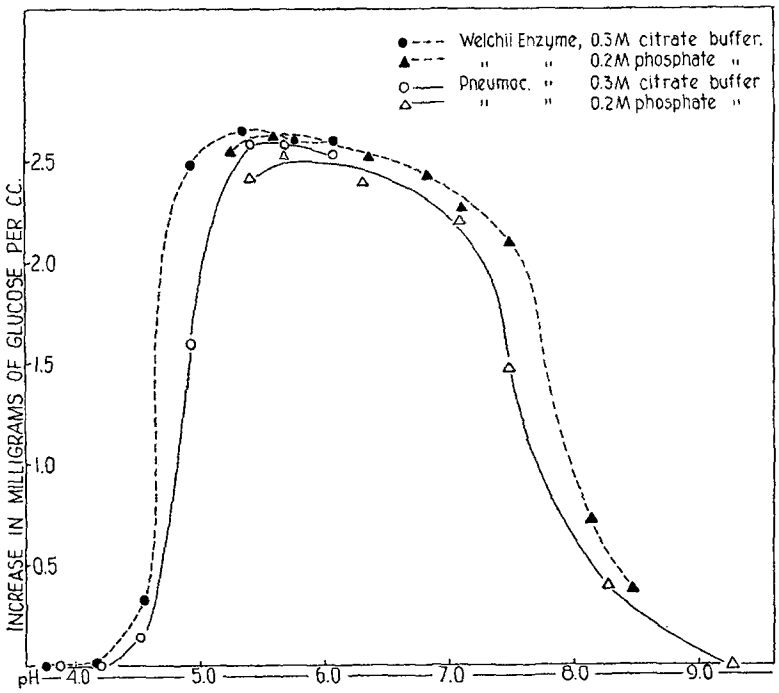

FIG. 1. pH activity curve of pneumococcus and *Cl. welchii* enzymes.

The hydrolysis of substrates other than hyaluronic acid by the enzyme preparations obtained from *Cl. welchii* is probably due to the presence of several different enzymes. Evidence in support of this belief is afforded by the observation that the various reactions exhibit different pH optima, and by the fact that the reactions do not run parallel in samples prepared by different methods. Table VI summarizes the range of activity of the enzyme preparations from pneumococcus, streptococcus, and *Cl. welchii* on various substrates. It can be seen from the table that the only substrate, other than hyaluronic acid, which was hydrolyzed by the pneumococcus and streptococcus enzymes was a trisulfuric acid ester of hyaluronic acid. The rate of hydrolysis of the latter was only 13 per cent of that of hyaluronic acid when compared in equimolar concentrations of the sub-

TABLE VI

Effect of the Bacterial Enzymes on Various Substances

Substrate	Carbohydrate components	Enzyme from pneumococcus and streptococcus	Enzyme from *Cl. welchii*	Remarks
Hyaluronic acid from Vitreous humor (cattle and pig) Umbilical cord (human) Synovial fluid (cattle and human) Group A streptococcus Fowl leucosis	Glucuronic acid, acetyl-glucosamine	+	+	Stable against diastase, ptyalin, taka-diastase, emulsin, egg white lysozyme, mixtures of enzymes from various staphylococci, *E. coli*
Pregnandiol β-glucuronide Borneol β-glucuronide	Glucuronic acid	0	0	
Starch, glycogen	Glucose	0	+	
Native and soluble chitin	Acetyl-glucosamine	0	0	
Ovomucoid-α	Glucosamine, mannose	0	0	
Serum mucoid	Glucosamine, mannose	0	0	
Soluble specific substance* Type I, II, III, XIV pneumococci		0	0	
C substance from pneumococcus*		0	0	
Neutral polysaccharide from pig gastric mucosa	Acetyl-glucosamine, galactose	0	+	
Chondroitin-sulfuric acid	Acetyl-chondrosamine, glucuronic acid, sulfuric acid	0	+	
Mucoitin-sulfuric acid from pig gastric mucosa	Acetyl-glucosamine, glucuronic acid, sulfuric acid	0	+	
Heparin†	Acetyl-glucosamine, glucuronic acid, (?) sulfuric acid	0	0	
Hyaluronic acid trisulfuric ester (synthetic)‡	Acetyl-glucosamine, glucuronic acid, sulfuric acid	+	?	
Acetyl-glucosamine Glucuronic acid		Unchanged		

* Obtained from Dr. M. Heidelberger.

† Crystalline barium salt, obtained from Dr. A. F. Charles.

‡ This substance was prepared by first sulfonating with solid SO_3, introducing one H_2SO_4 group. Two more sulfuric acid groups were introduced by the action of sulfonyl-chloride in pyridine at 100°. The substance had an anticoagulating effect of about 5 per cent of that of the crystalline heparin of Charles and Scott (15). (We thank Dr. E. Chargaff for the determination of the anticoagulating action.)

strates. Interestingly enough, heparin, which is supposed to have a similar structure (13, but see 14), was not attacked at all. Attention is called to the apparent absence of enzymes in the preparations from pneumococcus as well as from *Cl. welchii* directed against the specific carbohydrate of Types I, II, III, and XIV and against the C substance from pneumococcus.

C. Occurrence of the Enzyme in Splenic Tissue—In a previous communication (16) it was mentioned that an enzyme capable of hydrolyzing hyaluronic acid could be demonstrated in the ciliary body and iris of rabbits. A more potent source, and one which may possess greater biological significance, is splenic tissue. While studying the effect of β-glucuronidase from spleen on β-glucuronides, the observation was made that such an enzyme preparation also hydrolyzed hyaluronic acid. However, it is obvious that the two enzymes are not identical because emulsin does not hydrolyze hyaluronic acid while it does hydrolyze β-glucuronides. The occurrence of the enzyme in various tissues of the animal body and the possible rôle which it may play in hemolytic streptococcal infections is being studied further.

Relation of the Hydrolyzing Enzyme to the Bacteriolytic System of Pneumococcus

It has been shown (17) that living pneumococcal cells contain a group of enzymes, the bacteriolytic system, capable of causing the lysis of heat-killed pneumococci. Dubos (18) has shown that lysis first expresses itself by a loss of the Gram staining reaction and he has succeeded in partially purifying an enzyme responsible for this initial change. Dubos has further suggested that the enzyme responsible for the change in the Gram reaction of pneumococci is also capable of hydrolyzing hyaluronic acid. The reasons for this suggestion were as follows: "The agents responsible for both types of action are heat-labile, have the same pH optimum, are precipitable by flavianic acid, are reversibly inactivated by iodine, and finally, the action on pneumococci is inhibited by previous incubation of the ferment solution with sufficient amounts of the amino polysaccharide."

From data which have been obtained in the present study it appears unlikely that the hydrolyzing and bacteriolytic enzyme systems are identical. In the preparation of the carbohydrase fraction it was noted that the flavianate contained only a small amount of the lytic factor. Such preparations were highly active against hyaluronic acid. On the other hand, it was observed that the isoelectric fraction contained the greater part of the lytic factor and was very poor in carbohydrase. The total hydrolysis by the isoelectric fractions in 0.25 per cent concentration seldom reached 10 per cent of the theoretical reducing value after 20 hours, whereas

a concentration of 0.01 per cent of the flavianate effected 90 per cent hydrolysis in 20 hours. The isoelectric fractions in concentrations of 0.001 to 0.01 per cent invariably produced a change in the Gram reaction with subsequent lysis of the bacteria while the flavianate produced little or no change in the Gram stain. In all of these experiments the vaccines for the demonstration of the Gram stain were prepared according to the technique recommended by Dubos.[2]

Additional evidence for the belief that the factor responsible for the change in the Gram stain was not the same as the carbohydrase was obtained from the study of enzymes prepared from group A hemolytic streptococci and *Cl. welchii*. Although these preparations were highly effective in causing hydrolysis of hyaluronic acid, they failed to produce any change in the Gram reaction of pneumococci. It therefore seems unlikely that the two enzymes are identical.

DISCUSSION

It has been shown (2) that group A hemolytic streptococci elaborate a mucopolysaccharide which appears to be identical with hyaluronic acid. It has also been shown that the elaboration of the mucopolysaccharide is associated with the growth phase of the organism. Relatively large amounts may be obtained from encapsulated organisms in the mucoid phase and little or none from unencapsulated organisms in the rough phase. There is, furthermore, considerable indirect evidence that the attributes of virulence and invasiveness are closely associated with the presence of the mucopolysaccharide. Seastone (19) has recently brought forward convincing evidence in support of this view in the case of group C hemolytic streptococci and the same is probably true of group A organisms as well.

Preliminary experiments have suggested that the disappearance of the mucoid capsule of group A hemolytic streptococci may be due to the enzymatic hydrolysis of the polysaccharide. Highly effective enzyme preparations have been obtained from a strain (H44) which does not produce mucoid colonies and which is relatively avirulent for mice. On the other hand similar preparations from another strain (C203), which produces abundant mucoid growth and which is highly virulent for mice, appear to be lacking in enzymatic activity. Further experiments are now being carried out to determine more precisely the relationship between enzyme activity, the mucoid phase, and virulence of group A organisms. It is hoped to extend these experiments to include a study of the enzyme activity of the spleen and other tissues in different phases of infection.

[2] Personal communication.

SUMMARY

An improved method is described for preparing the enzyme which hydrolyzes the polysaccharide acid contained in vitreous humor, umbilical cord, synovial fluid, and the mucoid phase of group A hemolytic streptococci.

Preparations have been obtained from pneumococci, group A hemolytic streptococci, *Clostridium welchii*, and from splenic tissue, which display the same specific activity.

Evidence is presented to show that the hydrolytic enzyme is not the same as that responsible for the lysis of pneumococci.

In pneumococci and hemolytic streptococci the major portion of the enzyme is bound to the cell structure. The enzyme from *Clostridium welchii* is associated with other carbohydrate-splitting enzymes in the culture medium and not with the bacterial cells.

It is suggested that the disappearance of the mucoid capsule of group A hemolytic streptococci is due to enzymatic hydrolysis of the acid polysaccharide.

The relation between enzyme activity and the virulence and invasiveness of group A hemolytic streptococci is briefly considered.

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