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Hyaluronidase Activity of Bacteriophages of Group A Streptococci

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A sensitive dye-binding assay was employed to study the hyaluronidase associated with temperate and virulent phages infecting group A streptococci. Some enzyme was detectable in each purified phage preparation examined, but differences of several orders of magnitude separated the lower enzyme levels in virulent phages that required the addition of hyaluronidase for plaque formation and the higher levels in temperate phages that did not. Infection by virulent phage A25 was accompanied by the production of levels of hyaluronidase proportionate to the average burst size. Hyaluronidase was produced during infection by temperate phages at a much higher level than could be accounted for by the number of phage particles formed. The major portion of this hyaluronidase was free and apparently unassociated with phage or phage fragments. The phage-associated enzyme was tightly bound but could be released and solubilized by treatment with urea.

The penetration of the hyaluronic acid capsule by bacteriophage is a prerequisite for adsorption and productive infection of group A streptococci (7, 10). Strains of group A streptococci that possess a mucoid character because of such a capsule are resistant to infection by virulent phage, whereas such strains are susceptible to infection by many temperate bacteriophages (7). Kjems (7) demonstrated the appearance of the enzyme hyaluronidase in lysates of streptococci that had undergone infection with temperate phages and obtained evidence suggesting that a portion of this enzymatic activity constitutes an integral part of the phage particle. He showed that the hyaluronidase formed during phage production is distinct from the extracellular hyaluronidase produced by group A streptococci. Moreover, in these studies, the enzyme from several temperate phages showed immunological differences governed by the genetic makeup of the phages (7).

Since virulent streptococcal phage required the addition of hyaluronidase for plaque formation on mucoid strains (10), it was assumed from these earlier studies that hyaluronidase was absent in virulent phage. The development of a sensitive assay for hyaluronidase activity has allowed both a reexamination of this enzyme in association with virulent and temperate streptococcal bacteriophages and a study of the characteristics of its formation during infection as described in the present report.

MATERIALS AND METHODS

Strains. The temperate phages used in this study were obtained from group A, type 49 strains (University of Minnesota strains no. GT 8760 and GT 6484) by induction with mitomycin C as previously described (13). The virulent group A phage, designated as A25, was originally supplied by W. R. Maxted (11). The group A, M type 12 strain, designated as K56, was received from E. Kjems (7) and used for the growth of phages and as a standard indicator to prepare lawns. Bacteriophages were assayed as previously described (13).

Culture medium and preparation pf phage. Broth for phage and bacterial growth was modified according to the method of Malke (9) and contained 6% proteose peptone no. 3 (Difco Laboratories, Detroit, Mich.), 0.3% NaCl, 0.05% glucose, 5% horse serum (Grand Island Biological Co., Grand Island, N.Y.) and 2 mM $CaCl_2$. Agar plates for plaque assays contained 1% agar and 3% peptone only. Hyaluronidase (bovine testes; Sigma Chemical Co., St. Louis, Mo.) was added as indicated at a concentration of 40 μg/ml in plates.

To prepare bacteriophage stocks, overnight cultures of K56 (at 30°C) were incubated for 5 min at 37°C with phages at an input multiplicity of about 2 plaque-forming units per colony-forming unit and then diluted (1:100) into fresh medium. The phage multiplication was then allowed to proceed at 37°C for another 6 h. Thereafter, the lysate was chilled to 4°C, and cellular debris was removed by centrifugation at 8,000 × g for 30 min. The phage lysate was then incubated for 2 h at 37°C after the addition of pancreatic deoxyribonuclease I (10 μg/ml; Worthington Biochemicals Corp., Freehold, N.J.) and pancreatic ribonuclease (10 μg/ml; Sigma).

Concentration of phage. Phages were recovered from lysates by using the two-phase separation system of Albertson (1). The lysate was adjusted to contain (in final concentration) 2% NaCl, 0.2% sodium dextran sulfate (average molecular weight, 500,000; Sigma), and 7% polyethylene glycol (average molecular weight, 6,000 to 7,000; J. T. Baker Chemical Co., Phillipsburg, N.J.). After standing at 4°C for 16 h, the two phases separated. The lower phase was centrifuged for 15 min at 500 × *g*, and the pellet was resuspended in 0.01 M sodium phosphate buffer at pH 7.2 (one-fiftieth of the original volume of lysate). The suspension was adjusted to 0.3 M KCl by the dropwise addition of 3 M KCl and, after standing at 4°C for about 2 h, the suspension was centrifuged for 15 min at 500 × *g*. Phages were recovered from the supernatant fluid and sterilized by filtration through a membrane filter (HA type, 0.45-μm pore size; Millipore Corp., Bedford, Mass.).

Purification of phage. High-titer phage stocks were purified by equilibrium centrifugation on cesium chloride gradients. The gradients were prepared with 1.5-ml layers of CsCl solutions having the following densities (starting at the bottom of the tubes): 1.55, 1.46, 1.38, and 1.29 g/cm^3. A 10% sucrose solution (0.8 ml) in 0.01 M sodium phosphate buffer (pH 7.2) containing 1 mM $MgSO_4$ and 20 μg of deoxyribonuclease was layered on top of the CsCl gradient followed by layering of 1.0 ml of the phage suspension. After centrifugation at 100,000 × *g* for 1 h in a Spinco type 50 rotor at 4°C, phages were collected from the visible band and dialyzed overnight against 0.01 M phosphate buffer at pH 7.2.

Preparation of extracts of phage-infected bacteria. Overnight cultures of K56 (at 30°C) were infected with the respective phage (purified through CsCl) at multiplicities of infection of 2, diluted 1:10 into 100 ml of broth, and incubated at 37°C. At 0, 10, 20, and 30 min after infection, the cultures were poured over equal volumes of crushed frozen 0.01 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer at pH 7.2. Infected bacteria were then harvested by centrifugation at 8,000 × *g* for 30 min, washed twice with cold buffer, and suspended in 1 ml of physiological saline. The suspension was purified by layering bacterial cells located at the top 5-ml portion of 30% sucrose in 0.01 M Tris-hydrochloride (pH 7.2) on a 0.5-ml cushion of a saturated solution of cesium chloride. After centrifugation at 100,000 × *g* for 1 h in a Spinco 50 rotor at 4°C, the cells were collected from the interface of the CsCl and sucrose layers, frozen, partially thawed, and ground for about 10 min with alumina powder in a mortar at 4°C. The disrupted cells were suspended in 1 ml of 0.01 M Tris-hydrochloride, pH 7.2, and the suspensions were centrifuged at 30,000 × *g* for 2 h. The supernatant fractions thus obtained were filtered through membrane filters and dialyzed against 0.02 M acetate buffer (pH 5.0) containing 0.01 M NaCl before testing for hyaluronidase activity.

Protein content of the phage and bacterial host preparations was determined by the method of Lowry et al. (8), using bovine serum albumin as standard.

Hyaluronidase assay. The method used in this study utilizes a carbocyanine dye, 1-ethyl-2-{3-(-ethylnaphtho {1,2d}-thiazolin-2-ylidene)-2-methylpropenyl}naphto{1,2d}thiazolium bromide (catalogue no. 2718; Eastman Organic Chemicals Div., Eastman Kodak Co., Rochester, N.Y.) for the spectrophotometric microdetermination of hyaluronidase activity. The binding of hyaluronic acid to the carbocyanine dye results in a shift in the visible absorption of the dye, with a maximal absorbance at 640 nm (2, 3). The absorbance at 640 nm is quantitatively related to the amount of polysaccharide present (2). The breakdown of hyaluronic acid catalyzed by the enzyme is accompanied by a decrease in absorbance due to binding of dye (L. C. Benchetrit, S. L. Pahuja, E. D. Gray, and R. D. Edstrom, Fed. Proc. 35:1727, 1976). Unless stated otherwise, for routine assay of the enzyme, reaction mixtures contained the following components in a total volume of 10 μl: hyaluronic acid (human umbilical cord; Sigma), 1 μg; 20 mM sodium acetate buffer at pH 5.0 (reaction buffer); 10 mM sodium chloride; and appropriate dilutions of the high-titer phage stocks. Incubation was at 37°C, and the reaction was terminated by the addition of 0.09 ml of water. Dye (0.1 mM in water containing 50% dioxane, 0.01 M acetic acid, and 0.5 mM ascorbic acid) was added to the samples to a final volume of 1.0 ml, and optical densities were monitored at 640 nm with a model 240 Gilford spectrophotometer against a blank containing dye (0.9 ml) and water (0.1 ml). Activity was expressed in terms of an arbitrary unit that caused a 10% decrease in absorbance at 640 nm in 1 h.

RESULTS

Demonstration of the phage-associated hyaluronidase. The following experiment was designed to detect and quantitate hyaluronidase activity on the viral particle. Temperate phages isolated from a type 49, group A *Streptococcus* (strain GT 8760, University of Minnesota) were purified by CsCl gradient centrifugation, and hyaluronidase activity was measured. Figure 1 illustrates the kinetics of hyaluronic acid degradation during incubation with varying amounts of the temperate phage. No significant further decrease in absorbance resulted after 10 h when the reaction mixture contained phage equivalent to 130 μg of protein, suggesting that degradation was complete. The enzymatic cleavage of hyaluronic acid results in the formation of products that are unable to bring about the spectral change on addition of dye (L. C. Benchetrit, S. L. Pahuja, E. D. Gray, and R. D. Edstrom, Fed. Proc. 35:1727, 1976). Incubation of the reaction mixture for 24 h allowed the assay of hyaluronidase associated with a small amount of phage, an amount containing as little as 0.13 μg of phage protein, which represents approximately 10^4 plaque-forming units. Control experiments showed no measurable absorbance at 640 nm when the temperate phage alone was mixed with dye solution, indicating

an absence of interaction with the dye. When hyaluronic acid was incubated without enzyme for as long as 24 h at 37°C and then mixed with dye, no decrease in absorbance at 640 nm could be detected. The unit of activity was defined as that amount of enzyme which brings about a 10% decrease in absorbance of the dye-hyaluronic acid complex in 1 h under standard conditions. A linear relationship exists between the time necessary for degradation of 10% of the hyaluronic acid substrate and the log of the amount of enzyme (L. C. Benchetrit, S. L. Pahuja, E. D. Gray, and R. D. Edstrom, Fed. Proc. 35:1727, 1976).

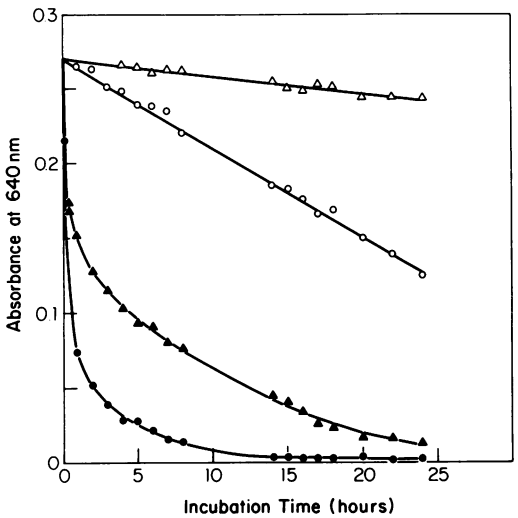

FIG. 1. *Kinetics of hyaluronic acid degradation by temperate phage hyaluronidase. Various portions of phages in 9 μl of 20 mM sodium acetate buffer (pH 5.0) containing 10 mM NaCl were added to 1 μg of hyaluronic acid solution (1 μl). After incubation at 37°C for various times, 90 μl of water was added to the reaction mixtures. The mixtures were kept at 4°C, and the dye solution (900 μl) was added for measurement of dye-complex formation at 640 nm. Phage protein; ●, 130 μg (equivalent to 10^7 plaque-forming units); ▲, 13 μg (10^6 plaque-forming units); ○, 1.3 μg (10^5 plaque-forming units); △, 0.13 μg (10^4 plaque-forming units). Phage was obtained from strain GT 8760 and propagated on K56.*

The hyaluronidase activities associated with two temperate and one virulent streptococcal phages are compared in Table 1. Generally, group A temperate phages do not require the addition of hyaluronidase to the medium in order to lyse the indicator strain K56 (7, 12). This observation is consonant with the relatively high level of hyaluronidase associated with the phage particles of most temperate phages, as exemplified by the temperate phage from strain GT 8760 in Table 1. A temperate phage was recently isolated from a group A strain of streptococci GT 6484 (13) that grows with low efficiency on the indicator strain K56 in the absence of added hyaluronidase. This phage possesses a level of enzyme approximately 2% of that of the temperate phage which is capable of infection in the absence of added enzyme (Table 1). Table 1 also indicates that hyaluronidase activity is associated with the virulent streptococcal phage A25 at a very low, but detectable level, using the dye-binding assay.

The addition of hyaluronidase does increase the efficiency of plating of both temperate and virulent phages. The enhancement is slight with the temperate phage from strain GT 8760. The lower level of hyaluronidase associated with the atypical temperate phage from strain GT 6484 is accompanied by an increase in the efficiency of plating in the presence of added hyaluronidase of almost two logs. With both of the temperate phages, the enhancement is minor compared with the effect of additional hyaluronidase on the infectivity of the virulent phage A25. With added enzyme, an increase of four orders of magnitude is observable in the titer of this virulent phage, which possesses a low, but detectable, level of hyaluronidase activity.

Hyaluronidase of phage lysate. All of the

TABLE 1. *Hyaluronidase contents of bacteriophages of group A streptococci and their relationship to phage titers with and without added hyaluronidase*

Phage	Hyaluronidase		Phage titer	
	Sp act		Added hyaluronidase	No added hyaluronidase
	U/mg of protein	U/PFU[a]		
Temperate phage				
From strain GT 8760[b]	62	8×10^{-7}	8.2×10^8	3.0×10^8
From strain GT 6484[c]	1.2	1.7×10^{-8}	2.1×10^8	4×10^6
Virulent phage A25	3.8×10^{-7}	2.7×10^{-16}	7.5×10^8	2.7×10^4

[a] PFU, Plaque-forming units.
[b] Propagated and assayed on K56.
[c] Obtained by spontaneous release and assayed lawns of strain GT 7907.

TABLE 2. *Distribution of hyaluronidase activity and protein in fractions of temperate phage lysate*[a]

Fraction	PFU[b]	Total protein (mg)	Hyaluronidase activity (U)	Sp act (U/mg of protein)
Sedimentable	0.9×10^8	1.2	75 (25%)	62
Non-sedimentable	Undetectable[c]	55.0	222 (75%)	4

[a] A culture of K56 (5 ml) was infected (multiplicity of infection, 0.1) with purified temperate phage (from strain GT 8760), and the lysate was centrifuged at 8,000 × *g* for 30 min. Thereafter, the supernatant was sterilized by filtration and centrifuged at 30,000 × *g* for 1 h. The phage pellet was resuspended in the reaction buffer (0.4 ml), and the supernatant was dialyzed against the same buffer. Enzyme activity was assayed in both fractions as described under Materials and Methods.

[b] PFU, Plaque-forming units.

[c] No plaques were observable when 0.03 ml of undiluted sample assayed for infectivity.

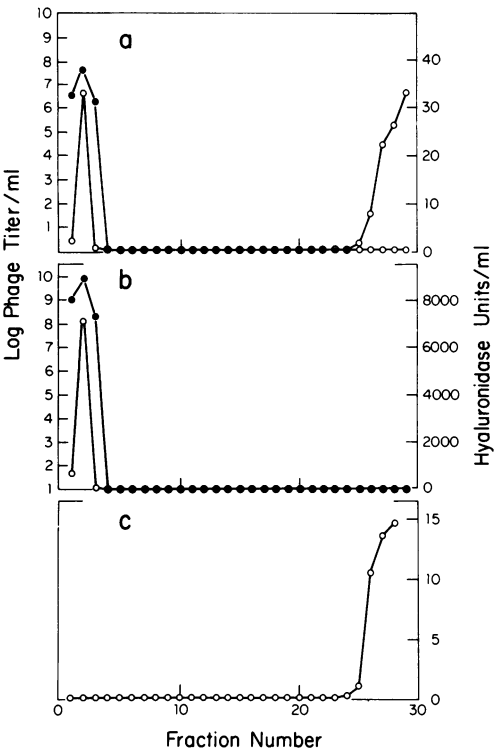

FIG. 2. *Sedimentation analysis of hyaluronidase in phage-induced lysates. Various lysate fractions obtained after infection of strain K56 with a temperate phage from strain GT 8760 were sterilized and layered on the top of 27-ml linear sucrose gradients (12 to 32%) prepared in 0.1 M NaCl upon a 1 ml cushion of 52% sucrose. (a) Whole lysate after removal of cellular debris by centrifugation at 8,000 × g for 30 min. (b) Temperate phage (purified through CsCl gradients). (c) Supernatant of lysate after removing of phages and cellular debris by centrifugation at 30,000 × g for 1 h. After centrifugation for 45 min at 20,000 rpm in a Spinco type SW25-1 rotor at 4°C, fractions of 1 ml each were collected, made 0.2 M with respect to sodium acetate (pH 5.0), and diluted (1:10), and 9-μl portions were assayed for enzyme activity or phage content.*

hyaluronidase activity present in temperate phage lysates is not associated with sedimentable phage. Table 2 presents the distribution of sedimentable (phage associated) and unsedimentable enzyme. Only 25% of the hyaluronidase in the lysate is sedimentable with phage particles. The non-sedimentable enzyme does not seem to arise by continual dissociation from phage, since subsequent purification of phage does not result in loss of hyaluronidase.

To determine whether the non-sedimentable hyaluronidase was associated with phage tails or other incomplete phage particles, the experiment shown in Fig. 2 was performed. Sucrose density gradient sedimentation analysis indicates that a whole lysate contains a peak of enzyme activity (28%) coincident with infectious phage particles (Fig. 2a). A second peak of enzyme activity (72%) remained at the top of the gradient. Analysis of previously sedimented phage demonstrates only phage-associated enzyme (Fig. 2b), and the supernatant of a lysate from which phage was sedimented contained only the soluble enzyme (Fig. 2c). There appears to be no indication that the non-sedimentable hyaluronidase activity is part of incomplete phage. The enzyme not associated with phage particles is free or at least bound in aggregates of relatively low molecular weight.

Hyaluronidase activity during phage infection. It was shown by Kjems (7) that infection of group A streptococci with temperate group A phages brings about the formation of hyaluronidase. This formation occurs in the phage-host bacterium system concomitant with the increase in the number of phages. It was of interest to demonstrate the hyaluronidase activity in extracts of phage-infected bacteria and to determine whether the phage controls the synthesis of the enzyme.

In the following experiment, exponentially growing K56 cells were infected with the indicated phages. At various times during the latent period, bacteria were harvested and

washed, and extracts were prepared as described under Materials and Methods. Hyaluronidase was assayed in these extracts, and the time course of enzyme formation expressed in terms of specific activity is illustrated in Fig. 3. Hyaluronidase activity is present in extracts of bacterial cells infected with the temperate phage (obtained from the M type 49, strain GT 8760, grown on K56) and with the virulent phage A25 (propagated on K56). It is also detectable at low levels in uninfected cells, although strain K56 produces a hyaluronic acid capsule. Formation of hyaluronidase was induced by both bacteriophages soon after infection. Enzyme levels continued to increase throughout the latent period, achieving a 10-fold increase for the virulent phage A25 and a 500-fold increase in specific activity for the temperate phage. Essentially no increase in enzyme specific activity was observable in uninfected cells. The differential formation of hyaluronidase indicated by these increases in specific activity during infection with either virulent or temperate phage is compelling evidence for phage control of this function.

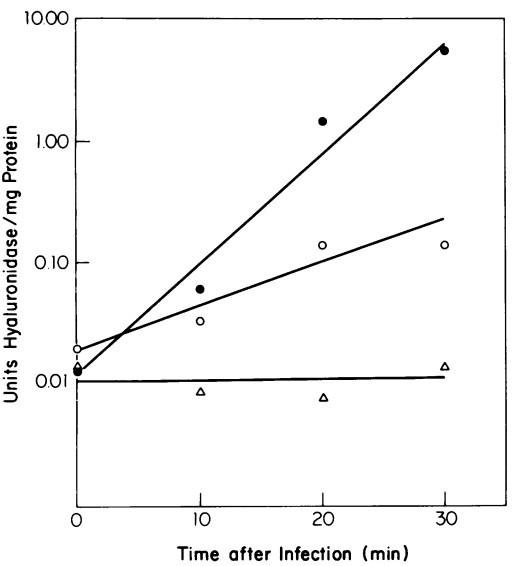

FIG. 3. *Hyaluronidase activity in streptococcal strain K56 infected with temperate and virulent phages. Cultures of K56 were infected at 37°C, and samples were withdrawn at various times (times: 0, 10, 20, and 30 min postinfection). Extracts were prepared from cells infected with the temperate phage (from strain GT 8760, ●) and from bacteria infected with the virulent phage A25 (○). Extracts were prepared from noninfected K56 at the times indicated when grown at 37°C (△). Reaction mixtures contained 1 μg of hyaluronic acid, 9 μl of cell extract, and reaction buffer in a total volume of 10 μl.*

TABLE 3. *Solubilization of the hyaluronidase enzyme by urea*[a]

Phage treatment	% Total activity	
	Supernatant	Pellet
Urea[b]	87	0
Buffer[c]	0	100

[a] Phage suspensions (50 μg of protein) were treated at room temperature for 30 min. Final volume was 0.5 ml.

[b] Phages were treated with 8 M urea and centrifuged, and portions of the supernatant (dialyzed against the reaction buffer) and the pellet (resuspended in 0.5 ml of the buffer and dialyzed) were incubated with hyaluronic acid.

[c] Phages were treated with reaction buffer and centrifuged, and the activity was assayed in both the supernatant and the pellet. Incubation mixtures contained 9 μl of sample and 1 μg of hyaluronic acid in a total volume of 10 μl. After incubation at 37°C, the mixtures were processed as described under Materials and Methods.

Solubilization of phage-associated hyaluronidase. The fraction of hyaluronidase activity associated with temperate phage particles is not removed by exhaustive purification of the phage through sedimentation and CsCl gradient fractionation. Thus, it appears to be firmly bound or represent an integral part of the phage particle. The enzyme can be solubilized, however, by urea treatment as is demonstrated in Table 3. Suspension of temperate phage in 8 M urea results in the dissociation of hyaluronidase to a non-sedimentable form. The enzyme may still be associated with other phage proteins but, if so, the aggregate is too small to be sedimented by centrifugation that completely sediments intact phage. Control experiments did not show a loss of activity when phages were treated with reaction buffer, centrifuged, and resuspended in buffer. Urea has no irreversible denaturing effects on hyaluronidase since phage lysates treated with urea that is subsequently removed by dialysis have enzyme activity comparable to untreated phage suspensions (not shown).

DISCUSSION

The involvement of hyaluronidase in the infection of group A streptococci with bacteriophages has been well established (6, 7). As first suggested by the studies of Kjems (7), a portion of the enzyme is sedimentable with the phage particle. This portion was demonstrated to be either an integral part of the phage particle or firmly attached to it. After extensive purification, a constant level of enzyme is retained in infective particles, i.e., repurification of phage

by the same procedures does not alter the specific activity of hyaluronidase. The sensitive assay method employed in the present study indicated that hyaluronidase is also associated with virulent phage, which requires the addition of hyaluronidase for plaque formation. The virulent phage, A25, contains an extremely low level of enzyme, but during the latent period of infection, the intracellular hyaluronidase levels increase approximately 10-fold. The latent period persists for longer than the time studied (4), but it is of interest that the increase in hyaluronidase is in the general range of the burst size of 10 to 30 (4, 5). It is possible that, since the enzyme formed is proportional to the number of the newly formed phage particles, it may be mainly associated with the phage. During the latent period of infection with a temperate phage (from a type 49 host), the specific activity of the enzyme increases more than 500-fold, although the burst size of a phage derived from a lysogenic group A, type 12 strain is about 32 (6). Evidently an excess of hyaluronidase is formed, more than is associated with the phage particles. This conclusion is in accord with the observations of Kjems (7), who estimated that one-third of the hyaluronidase in phage lysates is phage associated. Using a different system of temperate phage and propagating strain, a similar proportion of free enzyme (75%) was observed in the present study. Kjems (7) suggested that the hyaluronidase activity in lysates that are not associated with sedimentable phage particles consists of both free enzyme and enzyme present on phage tail fragments. This latter fraction was estimated by measuring the proportion of hyaluronidase in a phage-free lysis supernatant that bound to added streptococcal cells. Another interpretation of these results could be that enzyme was bound to the bacteria, not by virtue of being attached to a phage tail fragment, but by direct affinity with hyaluronic acid on the streptococcal surface. In fact, most of the enzyme not sedimentable with phage may be free, as might be concluded from the disparately large amount of enzyme formed during infection. In the study reported here, sedimentation analysis of phage lysates also suggested that the supernatant hyaluronidase activity is not associated with incomplete phage.

The phage-associated hyaluronidase is tightly bound to the phage or may be an integral element of the phage, essential for penetration of host mucoid capsule. The hyaluronidase can be solubilized by urea treatment of the phage, a procedure that disrupts the particle and will allow the purification and characterization of this enzyme. Such studies are presently in progress.

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