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Observations on the Group C Streptococcal Bacteriophage and Lytic Enzyme System[1]

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ABSTRACT

FOX, EUGENE N. (La Rabida-University of Chicago Institute, Chicago, Ill.), AND MASAKO K. WITTNER. Observations on the group C streptococcal bacteriophage and lytic enzyme system. J. Bacteriol. **89**:496–502. 1965.—The phage-associated lytic enzyme of group C streptococci was assayed by measuring the solubilized portion of radioactive cell walls. By this sensitive assay system, the induced synthesis of the lytic enzyme was observed intracellularly during phage infection; at least half of the total enzyme was synthesized and remained intracellular during the eclipse period, and was then released with the liberation of mature phage. Lytic enzyme could be detected in only two of eight lysogenic strains during temperate-phage production after ultraviolet induction. Virulent phage purified by density-gradient centrifugation contained lytic enzyme presumably associated with the virus *per se*. No hyaluronidase was detected in association with the phage, nor was this enzyme induced during phage synthesis. Variant strains of group C streptococci, no longer serologically active, were isolated as phage-resistant mutants. These strains still adsorbed the phage, but without subsequent virus reproduction, indicating that the group polysaccharide was not the primary receptor for the virus.

The potent lytic enzyme induced during group C streptococcal bacteriophage infection was characterized with respect to the kinetics of appearance of activity after virus adsorption. With the aid of radioactive substrates, the muralytic enzyme was identified in minute amounts in intracellular preparations and in association with purified phage. Further information has also been obtained with mutant hosts on the mode of group C phage adsorption.

MATERIALS AND METHODS

Cultures. The group C host strains C-1 and 88 were isolated from the upper respiratory tracts of patients. The various lysogenic group C strains (see Results) were also isolated from human sources. All strains were stored at −70 C and cultured as needed in Todd-Hewitt broth (Difco) at 37 C. The presence of temperate phage was determined after ultraviolet induction; the procedure is described in the text. Strain 88 was the most susceptible indicator for those phage which were screened against a large number of group C streptococci. Strain 88 could not be demonstrated to be lysogenic on any of 18 strains of group C streptococci used as indicators.

Bacteriophage. A group C phage, ϕY, was isolated as a mutant virulent virus from a lysogenic culture. This phage produced clear plaques, 1 to 5 mm in diameter, and induced lytic enzyme in strains C-1 and 88. The bacteriophage were assayed by plaque counting on soft agar-layer plates (Adams, 1959). A base of Heart Infusion Agar (Difco) was overlaid with Todd-Hewitt broth containing 0.7% agar, and a final volume of 7% defibrinated sheep blood was added to the soft agar just prior to pouring at 47 C.

Assay of the lytic enzyme. The activity of the lytic enzyme was measured by the extent of liberation of soluble radioactive material from cell walls obtained from group A streptococci grown in the presence of acetate-2-H^3. The rapid incorporation of acetate into amino sugars by streptococci was demonstrated by Dorfman et al. (1955). To prepare the walls, a 500-ml culture of streptococci in the exponential growth phase was supplemented with 4 mmoles of sodium acetate-2-H^3 containing 7.5×10^9 dpm. The culture was incubated for another 4 hr, and the cells were harvested and washed three times in buffered saline (0.01 M potassium phosphate in 0.9% NaCl). A 20% suspension of cells was ruptured by sonic treatment for 30 min in a Raytheon 9-kc sonic oscillator, followed by two freezing and thawing cycles. The cellular debris (about 500 mg, wet weight) was collected by centrifugation at 8,000 × *g* for 30 min and combined with about 2 g of unlabeled

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cell walls prepared in the same manner. The combined preparations were washed twice in 100 ml of buffered saline, and a 5% suspension in 0.1 M potassium phosphate buffer (*p*H 8.0) was incubated for 18 hr at 37 C with 0.1% crystalline trypsin; the *p*H was maintained at 8.3 by occasional titration with 1 M NaOH. A few drops of toluene were added as a preservative. After the trypsin digestion, the cell walls were washed three times in 100 ml of buffer and suspended at a concentration of 5% in 0.1 M acetate buffer containing 0.15 M NaCl, *p*H 5.5. The suspension (about 50 ml) was incubated with 2,000 units of highly purified testicular hyaluronidase (20,000 IU/mg) for 4 hr at 37 C. The walls were centrifuged at 3,000 × *g* for 15 min, and the sediment was discarded. The supernatant fluid was centrifuged at 10,000 × *g* for 30 min. The sediment was collected, suspended in 10 ml of buffered saline, and stored at −25 C. No radioactivity was released from the cell walls after further incubation with additional hyaluronidase. For use, a sample was thawed, washed twice at 10,000 × *g*, and diluted to the appropriate optical density. All assays of the lytic enzyme were performed in 0.1 M potassium phosphate buffer (*p*H 6.2) containing 0.001 M 2-mercaptoethanol. In most assays, 1 ml of a cell-wall suspension adjusted to a density of 150 Klett photometer units (66 filter), equivalent to 200 μg of walls per ml, was mixed with 1 ml of lytic enzyme solution in a thick-walled glass centrifuge tube. The mixture was incubated at 37 C for 1 hr, chilled, and centrifuged at 10,000 × *g* for 20 min. The supernatant liquid was carefully drawn off, and samples of this were assayed for radioactivity in an aqueous ethanol solvent system (Markovitz, Cifonelli, and Dorfman, 1959) in a Packard liquid scintillation spectrometer.

Concentration and purification of bacteriophage. High-titer phage preparations were obtained from confluent plaques by harvesting the soft-agar layer from Pyrex baking dishes (18 × 30 cm). The soft agar from each dish (20 ml) was washed from the surface layer with an equal volume of broth and extruded through a fine-mesh screen. The agar particles were removed by centrifugation at 5,000 × *g* for 15 min, resuspended with 10 ml of fresh broth, and again centrifuged. The combined supernatant fluids from six plates were concentrated to about 50 ml in dialysis tubing surrounded with flakes of Carbowax M20. A total of 50 ml of concentrated lysate contained about 5×10^{11} plaque-forming units per ml. Deoxyribonuclease (500 μg) and 0.001 M $MgCl_2$ were added to the concentrated phage suspension and incubated at 37 C for 60 min. The bacteriophage were centrifuged in 13-ml tubes for 120 min at 40,000 × *g* in a Beckman model L ultracentrifuge. After discarding the supernatant fluid, the pellets were overlaid with 2 ml of buffered saline containing 20 μg of deoxyribonuclease, 20 μg of ribonuclease, and 0.001 M $MgCl_2$. The pellets were gently fragmented with a stirring rod, and the suspension was kept at 5 C for 18 hr. Small fragments of debris were again gently broken, and the suspension was centrifuged at 5,000 × *g* for 30 min to remove unsuspended particles. The phage were again pelleted and suspended in a final volume of 12 ml of buffer. Cesium chloride was added to a concentration of 0.55 g/ml, and the tubes were centrifuged at 5 C at 100,000 × *g* for 24 hr in the Spinco no. 40 angle head rotor. (This gradient centrifugation procedure has been described by Strauss and Sinsheimer, 1963). After the centrifugation, the tubes were removed and the visible band of virus was removed dropwise through a hole punched in the bottom of the tube.

Hyaluronidase assay. A sensitive technique for assaying fractions of a unit of streptococcal hyaluronidase was developed with radioactive hyaluronic acid as a substrate. The hydrolyzed products, shown by Linker, Weissman, and Meyer (1954) to consist of disaccharide fragments of glucuronic acid and *N*-acetylglucosamine, were separated from undigested polysaccharide on Sephadex-G75. (We thank Alvin Markovitz for a purified preparation of streptococcal hyaluronic acid labeled with C^{14}-acetate with a specific activity of 40,000 counts per min per mg.) The column was a medium-grade beaded Sephadex-G75 (1 by 42 cm) equilibrated with 0.1 M acetate buffer (*p*H 5.2) containing 0.9% NaCl. To the column were added 3 ml of a reaction mixture in acetate buffer containing 4 mg of labeled hyaluronic acid. Fractions (2 ml) were collected at a rate of 10 ml/hr.

RESULTS

Characteristics of the lytic enzyme assay system. With the tritium-labeled cell walls as a substrate, the assay of the lytic enzyme was several times more sensitive than the method of measuring the lysis of whole cells. Various dilutions of a fresh phage lysate were mixed with a limited amount of radioactive cell walls (Fig. 1a). The solubilized material, expressed as counts per minute per milliliter of reaction mixture, is plotted versus the amount of lysate. A 0.01-ml amount of lysate, i.e., the lytic enzyme from about 10^6 infectious centers, could be easily measured. With an excess of cell walls (Fig. 1b and c), increasing amounts of a concentrated lytic enzyme preparation are plotted versus soluble labeled cell-wall material. The lytic enzyme was a concentrated fraction obtained by premature lysis of infected cells (Fox, 1963). Under the conditions of the experiments illustrated in Fig. 1a, no further digestion was observed after 60 min at 37 C. With increased amounts of substrate and enzyme as in Fig. 1b, 120 min were required for completion. When an excess of lytic enzyme was reacted with varying amounts of cell walls, after a 60-min incubation period, the amount of digestion was directly proportional to the substrate concentra-

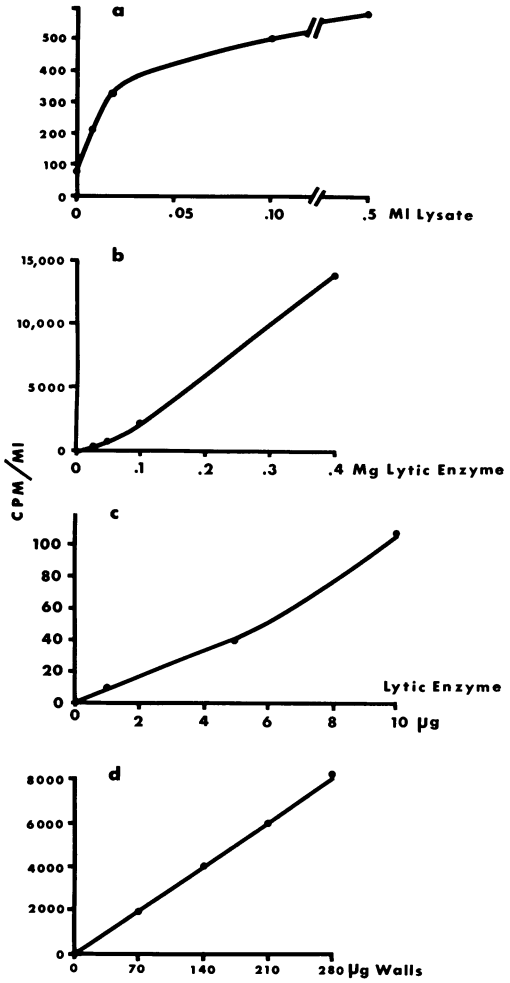

FIG. 1. *Radioactive assay of the lytic enzyme. (a) Fresh lysate added to a constant amount (200 µg) of labeled cell walls (see Materials and Methods). The amount of radioactivity in the solubilized supernatant fluid is measured. (b and c) Concentrated lytic enzyme reacted with an excess (600 µg) of walls. (d) Excess lytic enzyme reacted with increasing amounts of labeled walls.*

tion (Fig. 1d). Cell-free extracts of group C streptococci not infected with phage did not solubilize any radioactive material from the labeled walls.

Synthesis of lytic enzyme during phage infection. The rate of synthesis of the lytic enzyme was measured after phage adsorption to a susceptible host. With strain 88 in the exponential phase of growth, equal volumes of 10^9 cells per ml and 2.5×10^9 phage per ml were mixed at 37 C. To assay the extracellular lytic enzyme, 2-ml samples were removed at intervals and rapidly forced through a Millipore filter in a Swinny syringe adaptor. The filtered broth was added to a suspension of radioactive cell walls, and the activity of the lytic enzyme was assayed. The total lytic enzyme (intracellular plus extracellular) was determined by removing 5-ml samples from the cell-phage mixture at intervals and freezing them rapidly in prechilled tubes in a Dry Ice-acetone bath. Without prior concentration, the frozen samples were then crushed through a Hughes press by thawing the samples just enough to allow the frozen material to be removed from the tube to the receptacle of the press. After crushing at −40 C, the extruded material was thawed and centrifuged at 10,000 × *g* for 30 min, and the supernatant fluid was assayed for lytic enzyme as described. Samples were also removed from the original cell-phage incubation mixture at intervals and diluted into cold broth to assay the viable phage particles (Table 1). Between 10 and 15 min after adsorption (the total eclipse period), when over 99% of the phage particles were adsorbed, no extracellular lytic enzyme was detected. However, within 10 min after adsorption, intracellular lytic enzyme was observed, and after 15 min approximately half of the lytic enzyme produced was still intracellular; plaque assays indicated that only 10% of the new phage had been liberated after 15 min. Lysis was complete after 30 min, at which time the extracellular enzyme activity was the total activity of the sample.

Detection of lytic enzyme in lysogenic cultures. Eight lysogenic group C strains were tested for lytic-enzyme production after ultraviolet induction. The cells in the exponential-growth phase

TABLE 1. *Synthesis of the lytic enzyme during phage φY maturation*

Time after phage adsorption to strain 88	Plaque-forming units per ml	Lytic enzyme activity*	
		Total	Extracellular
min			
0	2.5×10^9	0	0
3	0.8×10^9	0	0
5	0.5×10^9	0	0
10	1.0×10^7	335	0
15	1.2×10^9	1,825	110
30	1.3×10^{10}	3,620	3,225

* Expressed in counts per minute per milliliter. Reaction mixture (1 ml) was added to 1 ml (200 µg) of tritiated cell walls. The amount of solubilized radioactivity per milliliter is recorded. Counts are corrected by subtraction of blank values (approximately 50 count/min) from samples containing only broth and labeled cell walls.

were washed and suspended in buffered saline to the original density and irradiated with a 15-w germicidal lamp for 30 sec at a distance of 50 cm and in a depth of liquid of about 2 mm. The irradiated cells were reinoculated into an equal volume of double-strength broth and incubated for 2 hr at 37 C. During this period, samples were filtered and assayed for phage and lytic-enzyme activity. Of these eight strains, only two produced lytic enzyme, and the amount was small when compared with virulent systems. Positive results were obtained with strain 9a with 88 as a control (Table 2). The first detection of the enzyme, 2 hr after irradiation, corresponded to the liberation of the temperate phage. The lytic enzyme could be detected only when the cells were broken in the Hughes press; apparently, not enough enzyme was liberated extracellularly to be detected in the filtered broth.

Lytic enzyme associated with phage particles. It was of interest to examine concentrated phage preparations freed of soluble lytic enzyme to determine whether the particles contained lytic enzyme activity analogous to the lysozyme of coliphage (Koch and Dreyer, 1958). Phage were purified and concentrated by sedimentation equilibrium in cesium chloride gradients as outlined under Materials and Methods. Two refractile bands were visible. The first, closer to the bottom, was removed from the tube as fraction 2 (Table 3), and the other, more bouyant and diffuse, was separated as fraction 4. The denser band, fraction 2, contained over 90% of the viable phage particles and a considerable amount of lytic enzyme activity compared to the fractions immediately above and below (Table 3). The enzymatic activity in fraction 4 was possibly attributable to phage-tail fragments However, no further work was done to identify the bouyant material.

TABLE 2. *Liberation of lytic enzyme after ultraviolet irradiation*

Time after irradiation	Temperate phage liberated		Lytic enzyme activity*	
	Strain 9a†	Strain 88	Strain 9a†	Strain 88
min				
0	−	−	168	152
30	−	−	200	
60	−	−	190	
120	+	−	351	140

* Expressed as counts per minute per milliliter. An excess of cell walls, 600 μg/ml, was used for the labeled substrate. Procedure was as described in Table 1. Blank values with excess substrate were 150 counts per minute per ml, ± 20%.

† Strain 9a was demonstrated to be lysogenic; strain 88 was the host.

TABLE 3. *Equilibrium centrifugation of phage φY in cesium chloride and association of enzyme with virus*

Fraction number, top to bottom of tube*	Vol of fraction	Plaque-forming units per ml (host strain 88)	Lytic enzyme activity†
	ml		
4	1.2	2.8×10^8	3,142
3	9.8	7.4×10^8	477
2	0.5	3.3×10^{11}	1,146
1	1.2	4.3×10^9	133

* See text for explanation.

† Expressed as counts per minute per milliliter. Lytic enzyme was assayed as described in Table 1.

Hyaluronidase activity. Kjems (1958) has demonstrated that hyaluronidase is a constituent of the group A streptococcal bacteriophage lytic process. He observed the production of the enzyme during lysis and also observed hyaluronidase in crude phage pellets collected by centrifugation.

In group C streptococci, the present data indicated that no hyaluronidase was demonstrable with phage φY and the two host strains. Initially, the turbidity method of Dorfman (1955) was used to assay hyaluronidase in phage lysates, infected cells broken in the Hughes press, and broth-culture supernatant fluids. In fresh cultures in the exponential phase of growth, traces of hyaluronidase were observed in the culture supernatant fluids, and that amount increased several-fold as the cultures aged. However, no additional enzyme was observed intracellularly or extracellularly during phage infection, nor was any hyaluronidase detectable on the phage particles. In order to substantiate this observation, an assay several times more sensitive than the turbidity method was devised with radioactive hyaluronic acid as a substrate. The disaccharide fragments resulting from enzymatic hydrolysis were separated from the undigested hyaluronic acid on a Sephadex G-75 column. The elution pattern of purified hyaluronic acid is compared with the patterns obtained with a crude phage lysate and 5×10^{12} phage purified by density-gradient centrifugation in cesium chloride (Fig. 2). The extent of enzymatic hydrolysis after 60 min at 37 C observed in a fresh phage lysate was no greater than that observed in a cell-free extract from an equivalent amount of uninfected cells. Purified phage, equivalent to a 100-fold concentration of the fresh lysate, contained no

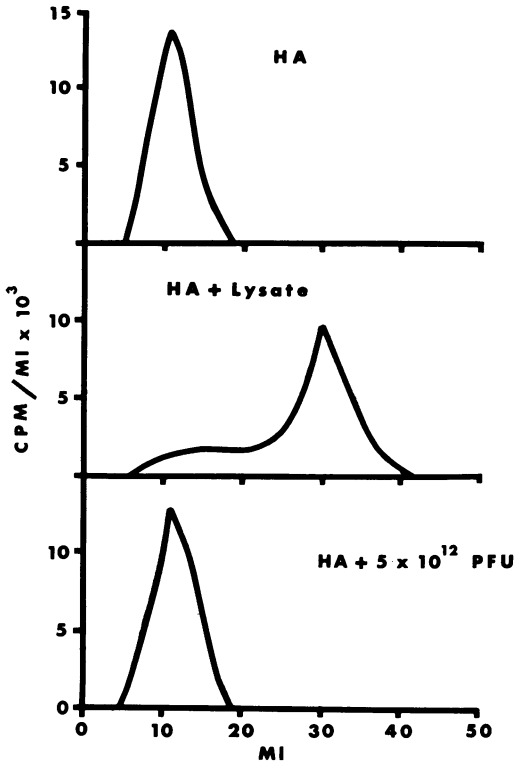

FIG. 2. *Separation of radioactive hyaluronic acid and hydrolysis products on Sephadex G-75.*

observable hyaluronidase activity; i.e., the enzyme was not associated with the phage particles, and, in addition, there appeared to be no significant increase in intracellular or extracellular enzyme activity during the virus life cycle.

Group C phage adsorption sites. A number of group C variant strains, i.e., mutants no longer serologically active, were isolated in this laboratory. These variants failed to produce serologically active group carbohydrate as a result of the loss of the terminal *N*-acetylgalactosamine residue of the polysaccharide (Araujo, Krause, and Fox, 1963). The two strains, C-31 and C-39, were isolated as phage-resistant mutants by replica plating of clones of strain C-1 on blood-agar plates spread with a heavy inoculum of phage ϕY. Approximately 4% of all phage-resistant mutants isolated, when extracted in hot HCl according to the method of Lancefield (1933), were no longer serologically reactive with group-specific antisera. Of several dozen serologically inactive isolates, only two remained so after repeated subculture, although all the mutants retained the phage-resistant character. Figure 3 is an agar immunodiffusion plate in which hot acid extracts of the wild-type, C-1, the two resistant mutants, and a control antigen were reacted with group C-specific antiserum (Difco). The two mutants lost the serological specificity of the C polysaccharide.

Previous studies (Krause, 1957) have shown that the host-range specificity of streptococcal bacteriophages is largely confined within serological groups; very few, if any, will infect hosts of more than one Lancefield group, and it has been assumed that the specific polysaccharide is the receptor site of group C streptococcal phages. With the C-variant strains isolated in this study, this hypothesis was tested; adsorption kinetics were performed with the aforementioned strains. In Fig. 4, the per cent of unadsorbed phage is plotted versus the minutes after mixing host and virus in a ratio of 10:1, respectively. To assay the free phage, samples were diluted 1:10 in cold buffer and filtered through Millipore membranes. The kinetics of adsorption were diphasic; about 90% of the phage were adsorbed within 2 min after mixing, both with the wild-type and the mutant hosts. The second phase of adsorption was delimited by the abrupt break in the curve, and, in the case of the resistant mutants, this second phase did not occur, and infection proceeded only in the wild type. These data indicate

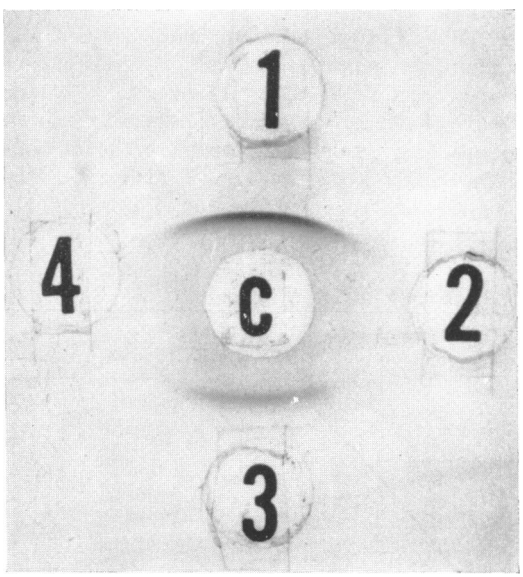

FIG. 3. *Immunodiffusion analysis. Well no. 1: acid extract of wild-type group C streptococcus; wells no. 2 and 4: extracts of C-variants, C-31 and C-39; well no. 3: group C antigen (Difco). In center well, C, is group C-specific antiserum. Protein was stained with light-green in 5% acetic acid.*

that the primary adsorption site of group C phage is not the group-specific carbohydrate.

DISCUSSION

With the aid of an accurate and sensitive assay system for the phage-associated lytic enzyme of group C streptococci, the intracellular induction of the enzyme during phage infection has been shown to be a comparatively early biosynthetic activity. It remains a moot question whether the lytic enzyme is necessary for the intracellular release of newly formed phage. These preliminary data indicate that infected cells remain intact with the lytic enzyme stored intracellularly. It may be speculated that other enzymes are also induced which are responsible for the dissolution of the cytoplasmic membrane; only after these have acted does the lytic enzyme attack the cell wall from within. In a *Streptococcus* phage infection with a multiplicity of 3 to 1, a fresh lysate becomes crystal clear at the end of a one-step growth cycle. This indicates that the cell membranes as well as the walls have been degraded. However, when cell walls are used as a substrate for the lytic enzyme, a portion of the wall remains undigested, and only when the walls are extensively treated with a surface-active agent such as Duponol (Barkulis and Smith, 1962) to remove fragments of membranes adhering to the walls will the lytic enzyme completely dissolve the fragments.

In the lysogenic cultures induced to produce temperate phage, six of eight strains tested did not form lytic enzyme at the time of phage liberation. None of the lysogenic group C strains "clear" after ultraviolet induction. The liberated phage produce pin-point plaques on the lawns of various indicator strains, and these plaques are only visible when formed confluently from a drop of undiluted supernatant broth. The size of these plaques, the lack of an appreciable amount of lytic enzyme, and the absence of clearing after lysogenic induction indicate the lytic enzyme is not necessary for the liberation of temperate phage in many group C streptococci.

The identification of lytic enzyme associated with the virulent phage ϕY is consistent with the observation of Kessler and Krause (1963), who demonstrated that sulfydryl-reacting reagents such as *p*-hydroxymercuribenzoate inhibit group A and C phages. Whether the lytic enzyme, a sulfydryl-dependent activity (Doughty and Hayashi, 1962), is required for streptococcal phage penetration is still not decided, although we have observed lysis-from-without at high multiplicities, implying an enzymatic mechanism of penetration.

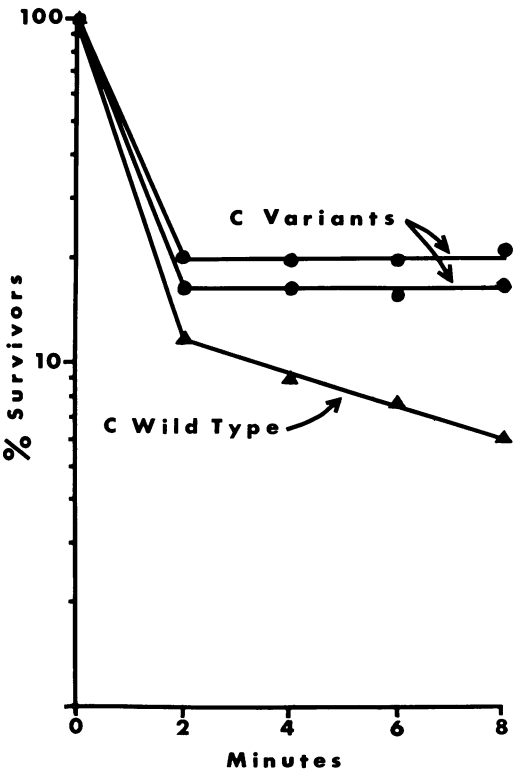

FIG. 4. *Rates of adsorption of phage ϕY to wild-type C-1 and resistant C-variants, C-31 and C-39. The per cent of unadsorbed phage are enumerated after the time of mixing with the various streptococci.*

Kjems (1958), working on group A streptococcal bacteriophages, claimed that hyaluronidase was a constituent of the phage particle and was also induced during phage growth. We have not been able to demonstrate the enzyme in association with the group C phage particles, nor have we demonstrated an induction of hyaluronidase during infection. A basal amount of enzyme was found intracellularly. Upon phage lysis, the enzymatic activity was largely associated with a pellet after ultracentrifugation. However, after phage purification no enzyme was demonstrable. It was possible that the enzyme was associated with fragments of membranes which were discarded after preliminary fractionation, and it is conceivable that a portion of the activity observed by Kjems in crude phage preparations was also associated with cell membranes.

It was observed by Krause (1957) that purified group C carbohydrate "inactivated" C-specific phages. The data presented here demonstrate that a secondary adsorption to host cells was apparently related to the presence of the anti-

genically specific group carbohydrate, but the primary adsorption was directed to other molecular groupings on the cell wall. Possibly, several closely spaced specific sites are required for the adsorption and subsequent infection by the virus, and the absence of one (e.g., group carbohydrate terminal amino sugars) still permits incomplete adsorption but no infection.

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