INFECTION AND IMMUNITY, Nov. 1972, p. 755-760
Vol. 6, No. 5

Properties of Purified Pneumococcal Hemolysin

MARY K. JOHNSON

Departments of Ophthalmology and Microbiology, Tulane University School of Medicine, New Orleans, Louisiana 70112

Received for publication 23 May 1972

With a purified preparation of pneumococcal hemolysin, a sigmoid relationship was found to exist between lysin concentration and hemolytic activity. Hemolysis was inhibited by a high ratio of erythrocytes to lysin, suggesting a multi-hit mechanism of action. Reaction rate decreased rapidly with time, possibly due to competition between ghosts and unlysed erythrocytes for fixation of lysin. The effects of *p*H and various agents on the processes of lysin adsorption and hemoglobin release were determined. The pneumococcal preparation did not possess nicotinamide adenine dinucleotide glycohydrolase activity.

Pneumococcal hemolysin is one member of a group of similar oxygen-labile, cholesterol-sensitive cytolytic toxins also including streptolysin O, clostridial θ toxin, and cereolysin. Although pneumolysin has been studied since the early part of this century, its mechanism of action remains unknown. The present report involves a study of properties of purified pneumococcal hemolysin carried out to confirm and extend previous observations made with crude material and to attempt to elucidate the mechanism of action of this lysin.

MATERIALS AND METHODS

Culture of bacteria. A smooth strain of *Diplococcus pneumoniae* (type 21 isolated from a corneal ulcer) was grown in clarified Trypticase soy broth (BBL) adjusted to *p*H 8.2. A 100-ml inoculum (grown overnight in this medium) was added to 900 ml of medium contained in a 1-liter Erlenmeyer flask and incubated at 37 C until the *p*H reached 5.8 (about 8 hr). The cells were harvested by centrifugation, washed twice with phosphate-buffered saline (PBS; 0.067 M potassium phosphate buffer, *p*H 7.0, with 0.077 M NaCl), and stored at −60 C.

Purification of hemolysin. A crude extract of cells (containing approximately 5 mg of protein/ml) was prepared by disruption of a suspension (10 g wet weight of cells plus 10 ml of water) in a French press, followed by centrifugation at 20,000 × *g* for 15 min. The extract was then fractionated essentially as described by Shumway and Klebanoff (9) involving acid precipitation, ammonium sulfate precipitation, and chromatography on diethylaminoethyl (DEAE)-cellulose and Sephadex G-100. Their procedure was slightly modified, however, in that elution from the DEAE-cellulose column was effected with a 0.05 to 0.2 M gradient of sodium phosphate buffer at *p*H 7.0 (hemolysin began to elute at 0.08 M), and a 10-fold concentration of the eluate fractions (to which was added 0.03 M β-mercaptoethanol) was accomplished by pressure dialysis. The filtration on Sephadex G-100 was performed with 0.05 M tris(hydroxymethyl)-aminomethane (Tris)-hydrochloride buffer at *p*H 7.5. This procedure produced a lysin with a specific activity of 450,000, representing a purification of 235-fold with a yield of 27%. Disc-gel electrophoresis (with 7.5% polyacrylamide at *p*H 8.3 and staining with Coomassie blue) of 0.2 ml of fivefold-concentrated Sephadex eluate showed a single band.

Purified hemolysin, with the addition of 0.1% bovine serum albumin (Fraction V, Armour), was stable at −60 C for at least 6 months.

Assay of hemolysin. Preparations were tested for hemolysin level by dilution in PBS containing 0.1% bovine serum albumin. (After the initial purification steps, preparations were activated before dilution by incubation for 15 min at room temperature in the presence of 0.03 M β-mercaptoethanol.) Appropriate amounts of diluted lysin were added to PBS in 12 by 100 mm tubes in an ice bath to a final volume of 1.5 ml. After thorough mixing, 1.5 ml of erythrocyte suspension was added. This was prepared from citrated rabbit blood by washing the cells twice with PBS and suspending them in PBS at a concentration which gave an optical density reading at 541 nm of 1.0 after dilution with an equal volume of PBS and addition of 0.1 ml of 1.0% saponin. The mixture was again thoroughly mixed and then transferred to a water bath at 38 C where incubation was continued for 30 min with frequent mixing. The mixtures were then chilled and centrifuged at 3,000 × *g* for 5 min to sediment the erythrocytes. The absorbance of the supernatant fluid was measured at 541 nm with a Gilford spectrophotometer.

The titer of the preparation (expressed as mean hemolytic dose [HD_{50}]) was estimated graphically, as the amount yielding 50% lysis, from a line constructed by using results of assay with three levels of lysin producing between 20 and 75% hemolysis. Specific activity was defined as HD_{50}/milligram of protein (determined by the method of Lowry et al. [7]).

Erythrocyte ghosts. Ghosts were prepared from freshly drawn rabbit blood by the procedure of Dodge et al. (3). The membranes from 8 ml of blood were suspended in 10 ml of 0.9% saline for tests on lysin fixation and release, which were performed by incubating 1 ml of ghost suspension with 300 HD_{5C} of lysin (an amount previously determined to be completely fixed by the ghosts) for 10 min at 37 C. The ghosts were then sedimented by centrifugation (20,000 × *g* for 15 min) and resuspended in 1 ml of distilled water. After subjection to the following procedures, the ghosts were resedimented, and the supernatant fluid was tested for the presence of released lysin: freezing and thawing five times; sonic disruption for 10 min; threefold dilution with distilled water; exposure (for 10 min) at room temperature to 1 M NaCl, 1 M urea, two volumes of butanol, two volumes of pentanol, acetate buffer at *p*H 4, Tris-hydrochloride buffer at *p*H 9.5, 0.01 M acetic acid, 0.03 M ethylenediaminetetraacetic acid (EDTA) (followed by fivefold dilution with distilled water or addition of HCl to *p*H 3.0 or addition of NaOH to *p*H 9), 0.001% sodium dodecyl sulfate. In all cases, concentrations of reagents carried over into hemolysin assays were not in themselves hemolytic.

Assay of nicotinamide adenine dinucleotide glycohydrolase (EC 3.2.2.5). Except as otherwise noted, the assay was performed as described by Fehrenbach (4). Seven-tenths micromole of nicotinamide adenine dinucleotide (NAD) (Sigma) was incubated with enzyme or lysin in 0.8 ml of 0.1 M Tris-hydrochloride buffer at *p*H 7.35. After incubation for 1 hr at 37 C, 3.0 ml of KCN was added, the the absorbance of the solution was measured at 325 nm. NAD glycohydrolase preparations from *Neurospora crassa*, pig brain, and calf spleen were obtained from Sigma Chemical Co.

RESULTS AND DISCUSSION

A sigmoid relationship between lysin concentration and extent of hemolytic activity (as measured by percent hemolysis) was observed (Fig. 1). Since the line is fairly straight between 20 and 75% hemolysis, points within this region were used for determination of HD_{50}.

On varying the erythrocyte concentration with a constant level of lysin, the percent hemolysis decreased with increasing erythrocyte concentration (Fig. 2). The absolute amount of hemolysis (measured by absorbance at 541 nm) increased to a maximum rate and then decreased, finally reaching a very low level. These results can be explained by a multi-hit requirement for lysis whereby each erythrocyte must fix a minimum number of lysin molecules greater than one before it can undergo lysis. In regions of high erythrocyte concentration and thus high erythrocyte to lysin ratio, few cells would have sufficient lysin for lysis to occur.

If collision between an erythrocyte and a lysin molecule results in fixation of the latter and the number of collisions is in proportion to lysin concentration, and a fixed number (>1) of lysin molecules is required for lysis, the Poisson probability model for random occurrence of collisions would lead one to predict a sigmoid relationship between lysin concentration and hemolytic activity, whatever the actual mechanism of cell damage. Such a relationship is depicted by Fig. 1.

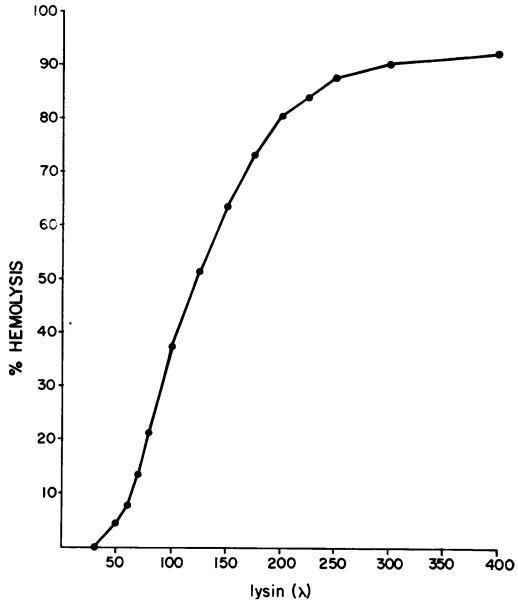

FIG. 1. *Effect of variation in lysin concentration upon the extent of hemolysis.*

The time course of the hemolytic reaction at various lysin concentrations is illustrated in Fig. 3. The reaction proceeds with a constantly decreasing rate, becoming very slow at a point where a large proportion of erythrocytes remain intact. Figure 4 shows that the shape of this curve is greatly altered by incubation at a lower temperature (25 C), in which case a distinct lag period was observed, followed by a brief period of increasing reaction rate which soon started to decrease as it did at the higher temperatures.

These results could be explained by a nonenzymatic reaction mechanism in which lysin is consumed. There are, however, several other possibilities compatible with an enzymatic mechanism.

The decrease in rate might result from the decreasing concentration of substrate (erythrocytes). However, addition of fresh erythrocytes to a lysed system resulted in no further lysis. The possibility of thermal inactivation must also be considered since the hemolysin is not very heat

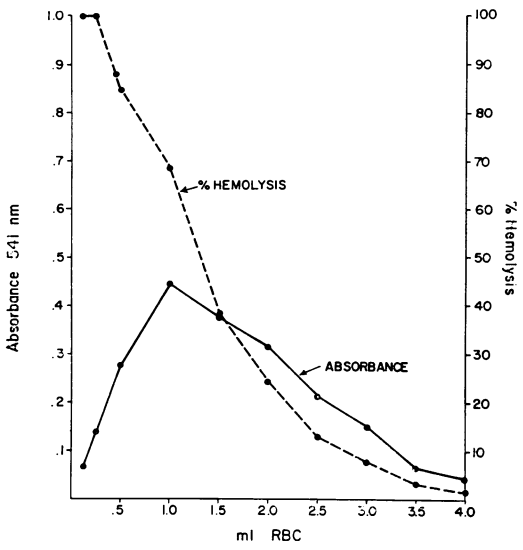

FIG. 2. *Effect of variation in erythrocyte concentration on hemolysis with a constant level of lysin (0.85 HD_{50}). The point at 1.5 ml of erythrocytes (1.5 ml RBC) represents the usual assay system.*

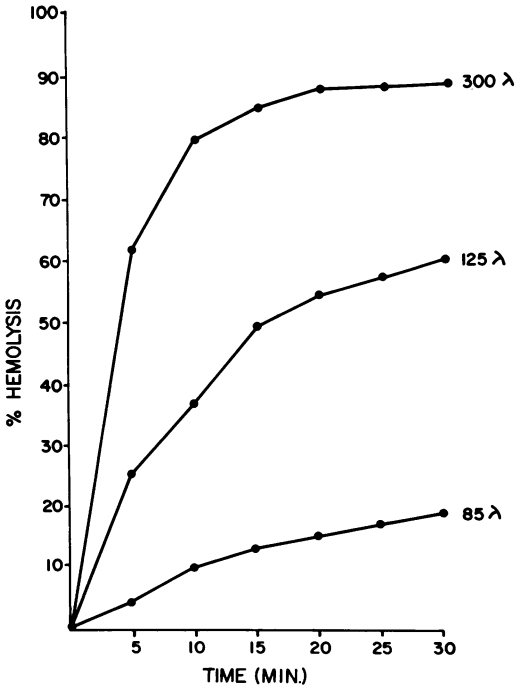

FIG. 3. *Time course of the hemolytic reaction at various levels of lysin (0.77, 1.13, and 2.73 HD_{50} [corresponding to 85 λ, 125 λ, and 300 λ] per 3-ml assay mixture).*

stable, being completely inactivated in 5 min at 50 C. However, lysin preincubated for 20 min at 38 C was found to react at the same rate as unheated lysin throughout a subsequent 30-min assay period. Inhibition of lysis by the products of hemolysis is another explanation of the decreasing reaction rate. Figure 5 shows the course of a control system and one to which hemolytic products were added at a concentration equal to that present at the end of a 30-min assay period with this level of lysin. The presence of lysate caused a distinct inhibition of hemolysis. When the lysate was treated by centrifugation at 20,000 × *g* for 20 min before the test, no inhibition was observed, probably due to the removal of erythrocyte ghosts. It is possible, then, that the observed decrease of reaction rate with time is caused at least in part by competition between unlysed erythrocytes and ghosts for fixation of lysin. The lysin-ghost bond would appear to be a very stable one as all attempts to release lysin adsorbed by ghosts (see Materials and Methods) were unsuccessful. An alternative explanation is that the ghosts (and the hemolyzing erythrocytes) in some manner inactivate the lysin.

On varying the *p*H of the assay mixture, activity decreased steadily between *p*H 6 and 8 (Fig. 6). That this effect is upon the process of hemoglobin release rather than on adsorption of lysin by the erythrocytes is shown by the data of Fig. 7. The effect of other agents on these processes is summarized in Table 1. Mn^{2+}, Ca^{2+}, and Zn^{2+} (but not Mg^{2+}) were inhibitory. The effect of Mn^{2+} was determined to be upon hemoglobin release rather than upon adsorption. EDTA had no effect. Parahydroxymercuribenzoate affected adsorption as one would expect, since sulfhydryl groups have been shown to be involved in this process. Cholesterol also affected only adsorption.

A recent report by Fehrenbach (5) presented evidence that streptolysin O (SLO) is an NAD glycohydrolase, cleaving the bond between nicotinamide and ribose in NAD. In tests for NAD glycohydrolase activity in the pneumolysin preparation (Table 2), none could be detected although the amount of lysin used was greatly in excess of a detectable level of activity if lysin activity and enzyme activity were in the ratio reported by Fehrenbach. NAD glycohydrolase activity was also tested in 0.1 M potassium phosphate buffer at *p*H 6 and *p*H 7.3 and with 0.2 ml of crude extract substituted for purified lysin; in all cases results were negative. *N. crassa* NAD glycohydrolase was used as a positive control. Lysin was added to the *N. crassa* preparation in an attempt to rule out the possibility of an enzyme inhibitor in the lysin preparation. The stimulation observed in that assay was shown to be due to

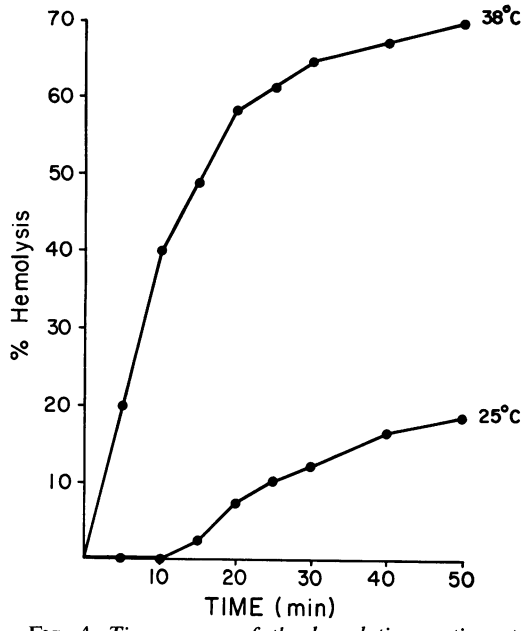

FIG. 4. *Time course of the hemolytic reaction at 25 and 38 C (1.15 HD_{50}).*

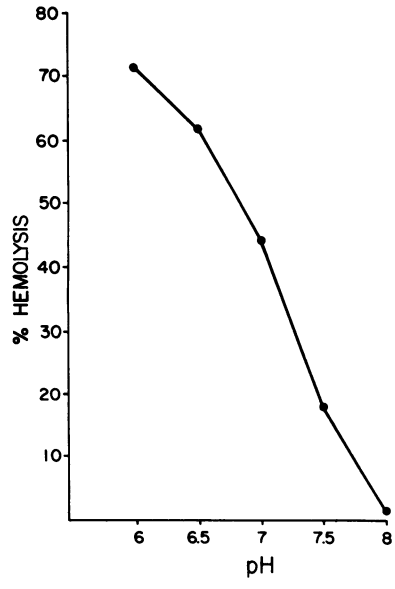

FIG. 6. *Effect of pH on hemolysis. The PBS used in the assay was adjusted to the indicated pH.*

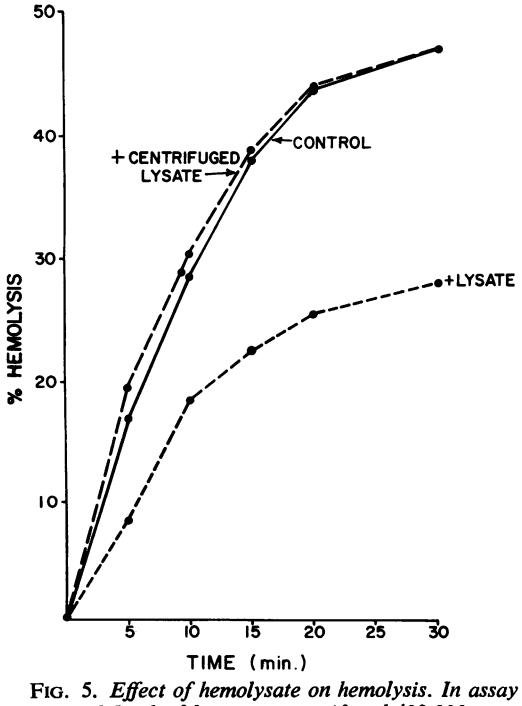

FIG. 5. *Effect of hemolysate on hemolysis. In assay mixture, 1.5 ml of lysate or centrifuged (20,000 × g for 20 min) lysate was added as indicated in place of the 1.5 ml of PBS used in the control system. The lysate was prepared from an assay mixture with 2 HD_{50} of pneumolysin incubated for 30 min, followed by sedimentation of unlysed erythrocytes.*

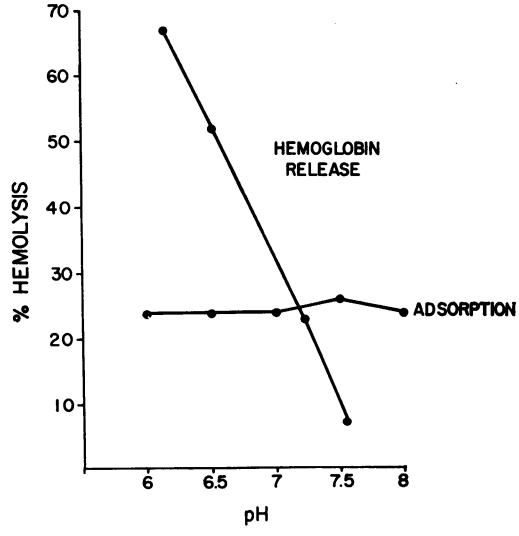

FIG. 7. *Effect of pH on adsorption and hemoglobin release. For tests on adsorption, complete assay mixture with buffer at indicated pH was held for 10 min in an ice bath, followed by sedimentation of erythrocytes, addition of 3 ml of PBS (pH 7), and incubation at 38 C for 30 min. For tests on hemoglobin release, the complete assay mixture with PBS at pH 7 was held for 10 min in an ice bath, followed by sedimentation of erythrocytes, addition of 3 ml of PBS at indicated pH, and incubation at 38 C for 30 min.*

TABLE 1. *Inhibitors of hemolysis*[a]

Agent	Concn (moles/liter)	Percent inhibition		
		Complete system	Adsorption[b]	Hemoglobin release[c]
$MnCl_2$	5×10^{-3}	90	0	97
$CaCl_2$	5×10^{-3}	27		
$MgCl_2$	5×10^{-3}	0		
$ZnSO_4$	1×10^{-3}	92		
Na_2SO_4	1×10^{-3}	0		
EDTA	1×10^{-3}	0		
p-Hydroxymercuribenzoate	1×10^{-6}	60		
	2×10^{-6}	100	100	0
Cholesterol	8.5×10^{-7}	81	70	0

[a] Assay mixtures contained 1 HD_{50} of pneumolysin.

[b] Inhibitor was added to complete assay mixture at 0 C for 10 min; cells were then centrifuged, resuspended in buffer, and incubated for 30 min at 37 C.

[c] Complete assay mixture was held for 10 min at 0 C; cells were then centrifuged, resuspended in buffer with inhibitor, and incubated for 30 min at 37 C.

TABLE 2. *NAD glycohydrolase activity*

Source of enzyme	Micromoles cleaved/hr	
	NAD	NADP
Pneumolysin (140 HD_{EC})	0	0
Neurospora crassa (0.02 enzyme units)	0.310	
N. crassa + pneumolysin	0.433	
N. crassa + 0.025% bovine serum albumin	0.420	

the presence of the bovine serum albumin in the lysin preparation.

Since nicotinamide is an inhibitor of some NAD glycohydrolases, the effect of this agent on hemolysis by pneumolysin was measured. An inhibition of 81% was observed only at the relatively high concentration of 0.1 M. (When tested separately, adsorption was inhibited 76% and hemoglobin release 12%.) Although some animal NAD glycohydrolases are more sensitive (e.g., the rat brain enzyme was 70% inhibited with 0.015 M nicotinamide), a relative insensitivity similar to that described here has been reported for the *Neurospora* enzyme (6) and for streptococcal NAD glycohydrolase (2).

Commercial preparations of NAD glycohydrolase from *N. crassa*, calf spleen, and pig brain were tested for hemolytic activity in the assay system used for pneumolysin at a level of 0.4 enzyme units, with negative results.

Many similarities can be noted in the properties of pneumolysin described in this paper and those reported in studies of SLO. Alouf and Raynaud (1) described a sigmoid relationship between SLO concentration and hemolytic activity and also noted inhibition of lysin by high ratios of erythrocytes to lysin. In addition, they showed data on time course of hemolysis similar to our findings with pneumolysin. Oberly and Duncan (8) working with SLO have reported differential sensitivity of the processes of adsorption and hemoglobin release similar to those described above.

In view of these similarities in the properties of the two lysins, one might expect a similar mechanism of action. None of the properties reported above for pneumolysin is inconsistent with an enzymatic mechanism. However, the NAD glycohydrolase activity which was reported by Fehrenbach to be associated with SLO (5) was not detected in the pneumococcal agent. Fehrenbach's evidence for the identity of SLO and streptococcal NAD glycohydrolase is very convincing. However, his findings are not in agreement with those of Carlson et al. (2) who reported separation of the two factors. If both activities are actually properties of the same molecule in the streptococci, then the evidence presented herein for lack of NAD glycohydrolase activity in our pneumococcal preparation suggests a quite different situation in this organism. The similarities in the properties of the pneumococcal hemolysin and SLO would have to be regarded as merely superficial. There is, in fact, another important difference in the properties of the two hemolysins in the frequently reported observation that pneumolysin is cell-associated whereas SLO is an extracellular agent.

ACKNOWLEDGMENTS

This investigation was supported by grant no. EY 00424 from the National Eye Institute. Expert technical assistance was provided by Flora Shoaf. I thank Timothy DeRouen for helpful discussions of statistical aspects of the problem.

LITERATURE CITED

1. Alouf, J. E., and M. Raynaud. 1968. Action de la streptolysine O sur les membranes cellulaires. II. Cinétique de la lyse érythrocytaire. Ann. Inst. Pasteur 115:97–121.
2. Carlson, A. S., A. Kellner, A. W. Bernheimer, and E. B. Freeman. 1957. A streptococcal enzyme that acts specifically upon diphosphopyridine nucleotide: characterization of the enzyme and its separation from streptolysin O. J. Exp. Med. 106:15–26.
3. Dodge, J. T., C. Mitchell, and D. J. Hanahan. 1963. The

preparation and chemical characteristics of hemoglobin-free ghosts of human erythrocytes. Arch. Biochem. 100:119–130.
4. Fehrenbach, F. J. 1971. Reinigung und Kristallisation der NAD-Glykohydrolase aus C-Streptokokken. Eur. J. Biochem. 18:94–102.
5. Fehrenbach, F. J. 1971. Identity of streptolysin-O and NAD-glycohydrolase (EC 3.2.2.5). Z. Naturforsch. Ser. B 26:1336–1338.
6. Kaplan, N. O., S. P. Colowick, and A. Nason. 1951. Neurospora diphosphopyridine nucleotidase. J. Biol. Chem. 191: 473–483.
7. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:263–275.
8. Oberly, T. D., and J. L. Duncan. 1971. Characteristics of streptolysin O action. Infect. Immunity 4:683–687.
9. Shumway, C. N., and S. J. Klebanoff. 1971. Purification of pneumolysin. Infect. Immunity 4:388–392.