PURIFICATION AND PHYSICAL PROPERTIES OF GROUP C STREPTOCOCCAL PHAGE-ASSOCIATED LYSIN*

By VINCENT A. FISCHETTI,‡ Ph.D., EMIL C. GOTSCHLICH, M.D., and ALAN W. BERNHEIMER, Ph.D.

(From the Department of Microbiology, New York University School of Medicine, New York 10016, and The Rockefeller University, New York 10021)

(Received for publication 15 January 1971)

It has been reported by Maxted (1) and Krause (2) that lysates of Group C streptococci infected with C1 bacteriophage contained an enzyme that had the ability to lyse Groups A, C, and E streptococci and their isolated cell walls. Crude and partially purified preparations of this enzyme were found to be useful for the isolation of Group A streptococcal cell wall components (M protein and C carbohydrate) (3–6), as well as for the preparation of cell wall-free protoplasts and protoplast membranes (7–11).

The first attempt to purify the Group C streptococcal phage-associated enzymes was by Krause (3) who, by repeated ammonium sulfate fractionations and high speed centrifugation, obtained a 100-fold concentration of the enzyme. Using a partially purified preparation of this lytic enzyme, Doughty and Hayashi (12) were able to determine that its activity was dependent on free sulfhydryl groups. Subsequently, Barkulis et al. (13), by repeated ammonium sulfate fractionations, high speed centrifugation, Sephadex G-100 gel filtration, and calcium phosphate adsorption and elution were able to purify the Group C phage-associated enzyme quite extensively. Using this enzyme preparation, they found a sedimentation coefficient of 5.53 and 5.03 S, a pH optimum of 6.1, as well as a dependence on monovalent cations for peak activity. They also confirmed the results of Doughty and Hayashi (12) that thiol-alkylating and mercaptide-forming reagents, as well as heavy metals, inhibited enzyme activity, while free -SH groups were essential for its action. By its sedimentation constant and the fact that the enzyme eluted immediately after the void volume of a Sephadex G-100 column, Barkulis et al. (13) estimated that the molecular weight of the lysin was in excess of 100,000.

Phage-associated lytic enzymes have been demonstrated in many other bacterial phage–host systems (14–19); however, only a few of these enzymes have been extensively studied (13, 17, 18, 20). Phage-associated enzymes in streptococci of Group D (21) and Group N (22) have also been reported. These two streptococcal phage enzymes have one characteristic in common with the Group C phage-associated enzyme

* Presented in part by Vincent A. Fischetti to New York University in partial fulfillment of the requirements for a Ph.D. degree.

‡ Present address: The Rockefeller University, New York, 10021.

i.e. a dependence on free -SH groups for activity, but no detailed comparison of the streptococcal enzymes has been carried out.

The marked instability of the phage lysin and its dependence on free -SH groups for activity may explain the relative lack of interest in the purification of this enzyme despite its potential usefulness as a tool for isolating streptococcal cellular fractions. The purpose of this communication is to describe a method by which the enzyme may be stabilized by the use of disodium tetrathionate, thereby allowing for a simplified method for preparing a highly purified lysin. Some of the physical properties of the purified phage lysin have also been explored.

Materials and Methods

Preparation of the C1 Phage Inoculum.—250 ml lf an 18 hr culture of Group C streptococcal strain 26RP66 was inoculated into 4 liters of Todd-Hewitt broth prewarmed to 37°C. The culture was allowed to grow at 37°C to an OD of 0.1 at 650 nm (in a 1 cm cuvette). 1 liter of C1 phage lysate containing 5×10^9 plaque-forming units (PFU)[1]/ml was added (the resulting phage to streptococcal chain ratio was 10:1) and the mixture was incubated until lysis occurred (45 min). This lysate was stored at 4°C for 4 wk to allow the residual lysin to become inactive, in order to avoid "lysis from without" when the lysate is added to the Group C cells for enzyme production.

Preparation of Crude Lytic Enzyme.—Crude, Group C phage-associated enzyme was prepared by modifications of the procedures of Markowitz and Dorfman (9) and Zabriskie and Freimer (23). 1 liter of an 18 hr culture of strain 26RP66 was inoculated into 10 liters of prewarmed Todd-Hewitt broth. Within 3 hr the exponential culture had attained an OD of 0.1 at 650 nm. 3 liters of C1 phage lysate containing 5×10^9 PFU/ml was then added to the streptococcal suspension and allowed to incubate for exactly 20 min. At this time, the phage-infected streptococcal culture was poured over ice to slow down phage replication and quickly sedimented in a Sharples Super-Centrifuge (Pennsalt Chemicals Corp., Warminster, Pa.). The cells were then resuspended in 60 ml of 0.05 M phosphate buffer, pH 6.1, containing 5×10^{-4} M dithiothreitol (DTT), and 5 mg bovine pancreatic deoxyribonuclease (Worthington Biochemical Corp., Freehold, N.J.). The suspension was then incubated at 37°C for 30–60 min during which time the cells lysed releasing the lytic enzyme. At this time, ethylenediaminetetraacetic acid (EDTA) was added to a concentration of 0.005 M. Centrifugation of the suspension at 27,000 *g* for 2 hr in a Sorvall SS-34 angle head (Ivan Sorvall, Inc., Norwalk, Conn.) removed most of the cellular debris. Disodium tetrathionate was then added to the enzyme-active supernatant to a final concentration of 0.3 M and gently mixed for 1 hr at 4°C, whereupon dry ammonium sulfate was added to 50% saturation. After 18 hr at 4°C, the lysin-containing precipitate was sedimented at 27,000 *g* for 1 hr, resuspended in 50 ml of 0.05 M phosphate buffer, pH 6.1, with 0.005 M EDTA, and dialyzed against 20 liters of this same buffer at 4°C. After dialysis, the lysin was centrifuged at 36,000 rpm for 5 hr in a No. 40 rotor of a Model L Spinco Preparative Ultracentrifuge to remove additional debris and most of the phage particles. The supernatant containing the crude enzyme was stored at −51°C until used. The final volume varied between 50 and 70 ml.

Preparation of Disodium Tetrathionate.—Disodium tetrathionate was prepared as described (24); however two to three recrystallizations of the material are essential in order to remove all

[1] Abbreviations used in this paper: DTT, dithiothreitol; PFU, plaque-forming units.

traces of iodine. The recrystallization process was repeated until the alcohol in which the precipitate formed was clear. The resultant powder was dried over calcium chloride and stored at 4°C under vacuum, in an amber glass bottle.

Reactivation of Tetrathionate-Inactivated Enzyme.—Reactivation of the phage enzyme inactivated with tetrathionate was accomplished by preparing an initial 1:10 dilution of the enzyme in 5×10^{-2} M DTT and allowing it to stand for 15 min at room temperature.

Preparation of Substrate for Testing Lysin Activity.—An 18 hr culture of Group A streptococcal strain $T25_3$ grown in Todd-Hewitt broth was centrifuged and washed once with 0.05 M phosphate buffer, pH 6.1, containing 0.005 M EDTA. The washed cells were then suspended in the same buffer to an OD of 0.6 at 650 nm on a Beckman spectrophotometer equipped with a Gilford No. 209 digital readout (Gilford Instrument Co., Oberlin, Ohio).

Assay for Lysin Activity.—Enzyme activity was measured by turbidimetric determination of cell lysis. The reaction mixture was composed of 1 ml of a suitable dilution of reactivated enzyme in 0.05 M phosphate buffer, pH 6.1, containing 0.005 M EDTA, plus 1 ml of the substrate cells. The mixture was then incubated at 37°C for 15 min at which time the OD at 650 nm was determined (in a 1 cm cuvette). One unit of lysin is defined here as that concentration of lytic enzyme which causes a 50% drop in optical density in 15 min at 37°C, as compared with the optical density of a control tube without enzyme.

Protein Determination.—Enzyme protein was determined by ultraviolet absorption at 280 nm.

Polyacrylamide Gel Electrophoresis.—Electrophoresis on polyacrylamide gels was carried out as described by Davis (25). Samples in 40% sucrose were layered over the upper gel and run at 1 ma/gel for 20 min, then 2.5 ma/gel until the bromophenol blue marker dye reached the end of the gels. Gels were stained with a 0.25% solution of Coomasie blue in 10% trichloracetic acid for 18 hr, then destained in distilled water. Some gels were tested for lysin activity immediately after electrophoresis by slicing them into 2.5 mm segments and placing each segment into a test tube containing 1.0 ml of 5×10^{-2} M DTT in 0.01 M potassium phosphate buffer, pH 6.1. Each segment was crushed with a glass rod and centrifuged. The supernatant was then assayed for lytic activity.

Preparation of Cellulose Phosphate.—Whatman cellulose phosphate (P11) was obtained from W. and R. Balston, Ltd., Maidstone, Kent, England. A suspension of the cellulose phosphate was prepared by mixing 70 g of powder with 1 liter of 1 M NaOH. After mixing for 1 hr, the suspension was allowed to settle and the fines removed by suction. The cellulose phosphate was then washed exhaustively with distilled water to remove the base, acetic acid was added to a final concentration of 2 M, and the mixture allowed to stand for 18 hr at 4°C. The suspension was again washed with distilled water in order to remove all traces of acid, then equilibrated in 0.1 M phosphate buffer, pH 6.1, containing 0.005 M DETA and 10% glycerol, and stored at 4°C in the presence of 0.02% sodium azide.

Molecular Sieve Chromatography.—The distribution coefficient (K_d) of the phage enzyme was determined by the method of Andrews (26). Sephadex G-100 was equilibrated in 0.1 M phosphate buffer, pH 6.1, containing 0.02 M EDTA and 10% glycerol, and poured into a 1.5×80 cm column. A flow rate of 8 ml/hr was used and approximately 1.0 ml samples were collected in preweighed tubes; the exact volumes were then determined gravimetrically. Dextran blue 2000 (Pharmacia Fine Chemicals, Uppsala, Sweden) and tritiated water (New England Nuclear Corp., Boston, Mass.) were used to determine the void volume and the total volume of the column, respectively. Two reference proteins, blue dextran, tritiated water, and the purified phage enzyme were mixed to a volume of 1.5 ml in the 0.1 M buffer and co-chromatographed at 4°C for each run on the G-100 column. Samples were tested for protein concentration by absorption at 280 nm; enzyme activity was determined by the assay described

above. Radioactivity was determined by liquid scintillation counting in a Beckman SS-133 counter using a fluor consisting of Bio-Solv BBS-3 (Beckman Instrument Co., Fullerton, Calif.)–Liquifluor NEF903 (New England Nuclear)–toluene (50:21:429) system. Blue dextran was assayed by absorption at 620 nm.

Sedimentation Coefficient.—Determination of the sedimentation coefficient of the enzyme was carried out by the method of Martin and Ames (27). Samples (0.1 ml) were layered onto a 2.55 ml linear sucrose gradient of 5–20% sucrose in 0.1 M phosphate buffer, pH 6.1, containing 0.02 M EDTA, and were centrifuged at 38,000 rpm for 17 hr at 4°C in a Spinco (Beckman) SW-39 rotor. 0.1 ml of lysin (40,000 units) and of the reference proteins (4 mg/0.1 ml) were run in separate tubes; however, for some experiments two reference proteins were run in the same tube. Fractions of seven drops (approximately 0.1 ml) were collected and assayed for lysin activity or protein content.

EXPERIMENTAL

Purification of Lytic Enzyme.—All purification steps for the phage lysin were carried out at 4°C.

Step 1: Tetrathionate Inactivation and Ammonium Sulfate Precipitation.—Crude phage enzyme was isolated, inactivated with tetrathionate, and precipitated with ammonium sulfate as described in Materials and Methods. This stock enzyme was used for purification.

Step 2: Cellulose Phosphate Chromatography.—A 1.5 × 20 cm column was poured with cellulose phosphate, equilibrated with 0.1 M potassium phosphate buffer, pH 6.1, containing 0.005 M EDTA and 10% glycerol, and 20 ml of the stock enzyme (from step 1) was applied. The column was then washed with the same buffer until the readings at 280 nm had reached baseline. The material which passed directly through the column under these conditions contained most of the contaminating protein, Group C carbohydrate and phage particles, while the enzyme remained on the column along with a small amount of unrelated protein. Since preliminary experiments had indicated that the enzyme eluted at 0.4 M NaCl in the 0.1 M phosphate buffer at pH 6.1 with EDTA and glycerol, the lytic enzyme was eluted in one step using this buffer system. The fractions containing the lytic activity, which corresponded to a small protein peak, were pooled.

Step 3: Calcium Phosphate Gel Adsorption and Elution.—In preliminary experiments it was found that the enzyme adsorbed completely to calcium phosphate gel when the molarity of NaCl in the solution was 0.2, while only partial adsorption was observed as the molarity approached 0.3 and above. Adjusting the molarity of the pooled fractions (from Step 2) to 0.2 was accomplished by reducing the concentration to one-half by adding 0.01 M potassium phosphate buffer, pH 6.1, containing 0.005 M EDTA and 10% glycerol. (When diluting the enzyme solution, however, a deduction of 1 ml for every milligram of protein in the solution should be made since this volume will be corrected when the calcium phosphate suspension is added). Calcium phosphate (Bio-Gel HTP, Bio-Rad Laboratories, Richmond, Calif.), washed and equilibrated in the 0.01 M buffer, was suspended at a final concentration of 30 mg/ml (dry weight) and added to the enzyme solution at a ratio of 30 mg of gel/milligram of protein. The mixture was stirred for 30 min and the calcium phosphate sedimented by centrifugation. The enzyme was then eluted from the gel by resuspending it in 6.0 ml of 0.1 M potassium phosphate buffer, pH 6.1, 0.005 M EDTA, and 10% glycerol containing 8.1 M NaCl. The solution was stirred for 15 min, the gel again sedimented by centrifugation; and the supernatant containing the enzyme was recovered. As well as offering a modest purification, this step served as a convenient method for concentrating the enzyme from the pooled fractions from step 2.

Step 4: Passage through Sephadex G-100.—The protein solution eluted from the calcium phosphate was applied to a Sephadex G-100 column (1.5 × 86 cm), equilibrated with 0.1 M

phosphate buffer, pH 6.1, containing 0.005 M EDTA and 10% glycerol. 1 ml fractions were collected and those with high lytic activity were pooled and stored at −51°C. Table I summarizes the results of the purification procedure in which the lytic enzyme was purified 652-fold with a recovery of 30% of the lytic activity.

Enzyme purification was monitored by polyacrylamide gel electrophoresis. Fig. 1 (*A*, *B*, and *C*) illustrates densitometric scans of stained polyacrylamide gels of samples of the crude enzyme (*A*) and two purification steps (*B* and *C*). As can be seen, a substantial purification resulted by passage through cellulose phosphate with subsequent adsorption and elution from calcium phosphate; however, one major and one minor protein peak were seen (Fig. 1 *B*). When a duplicate gel was cut into 2.5 mm segments and tested for lytic activity (as described in Materials and Methods), it was found that the enzyme was located

TABLE I

Purification of Streptococcal Phage-Associated Enzyme

Step	Procedure	Vol.	Concentration	Total units	Protein	Specific activity	Recovery	Purification
		(*ml*)	(*units/ml*)	(× 10^5)	(*mg/ml*)	(*units/mg*)	(%)	
	Crude enzyme	20	115,300	23.1	103.62	1,113	100	1
1	Tetrathionate and ammonium sulfate	20	76,886	15.4	19.80	3,883	66.6	3
2	Cellulose phosphate chromatography	23	76,886	17.7	0.34	226,135	76.0	203
3	Calcium phosphate	6	200,000	12.0	0.66	303,030	52.0	272
4	Sephadex G-100	15	45,000	6.7	0.062	725,806	30.0	652

in the slow moving peak (bar graph). After sieving through Sephadex G-100, most of the fast-moving contaminants were removed, leaving the enzymatically active peak as the major protein component (Fig. 1 *C*). By integrating the areas under the three peaks (Fig. 1 *C*), it was estimated that the enzymatically active peak contained 70% of the total protein of this final preparation.

Physical Properties of the Phage Enzyme

Gel Filtration of Lysin and Reference Proteins.—Reference proteins (bovine serum albumin, soybean trypsin inhibitor, ovalbumin, and ribonuclease) were used in various combinations with lysin, dextran, and ^3H_2O to determine their peak elution volumes on a Sephadex G-100 column. Fig. 2 is an example of one such run. From these peak elution volumes, the distribution coefficient

(K_d) was calculated. The K_d is defined here as the volume of solvent within the pores of the gel which is accessible to the solute. This parameter, described by Ackers (28), is further elaborated by Siegel and Monty (29).

In Fig. 3, the observed values of K_d are plotted *versus* the logarithm of the

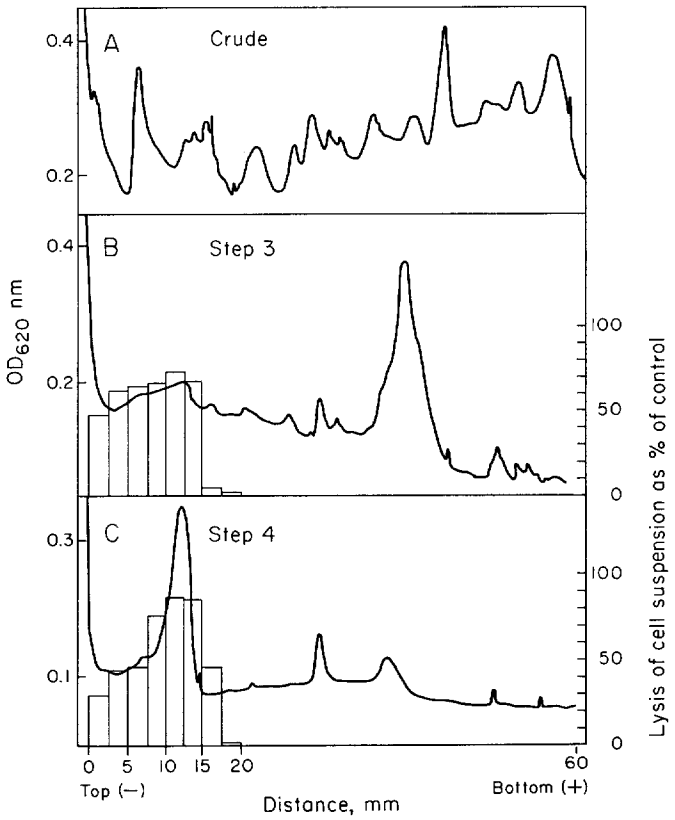

FIG. 1. Densitometric scans of stained polyacrylamide gels of (50 μg) samples (*A*) Sample after crude enzyme isolation, before tetrathionate inactivation and ammonium sulfate precipitation. (*B*) Sample after cellulose phosphate chromatography and adsorption and elution from calcium phosphate (step 3). (*C*) Sample after Sephadex G-100 chromatography (step 4). Duplicate gels from *B* and *C* were cut into 2.5 mm segments and tested for lytic activity as described in Materials and Methods (bar graph).

molecular weight of the reference proteins. As described by Andrews (26) and Whitaker (30), this method can be used for the estimation of the molecular weight of globular proteins with low frictional ratios. The observed K_d of the purified (step 4) lysin was 0.201 which, utilizing the reference line, corresponds to a molecular weight of 105,000. It is interesting to note that when 1.5 ml of

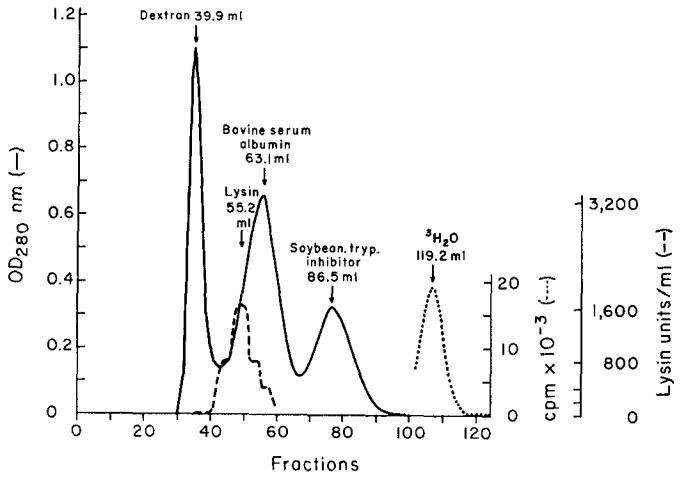

FIG. 2. Gel filtration of phage lysin with reference proteins on Sephadex G-100 equilibrated in potassium phosphate buffer, pH 6.1, containing 0.02 M EDTA and 10% glycerol. Blue dextran and tritiated water were co-chromatographed with the lysin and reference proteins to determine the void volume and total volume of the column. Fractions were tested for protein concentration, lytic activity, and radioactivity.

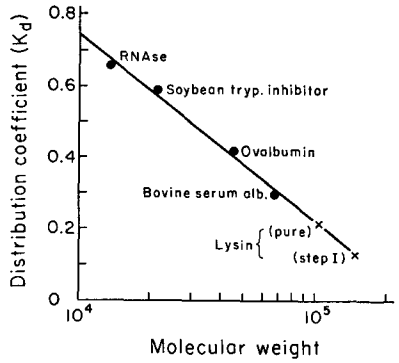

FIG. 3. The distribution coefficients (K_d) of the reference proteins were plotted *versus* the logarithm of their molecular weight. The observed K_d of the purified (step 4) lysine was 0.201 which corresponds to a molecular weight of 105,000. The K_d of the crude (step 1) enzyme was 0.128 corresponding to a molecular weight of $\geq$150,000. See text for calculation of K_d.

crude (step 1) lysin was passed through the same column under the same conditions as the purified enzyme, a K_d of 0.128 was consistently obtained, corresponding to a molecular weight of $\geq$ 150,000, which is beyond the resolution capacity of the G-100 column (26). After passage of the step 1 enzyme through the G-100 column, the enzymatically active peak was found to contain the Group C carbohydrate, as assayed by the capillary precipitin technique. These

results are suggestive of an enzyme–substrate complex causing an increase in the molecular weight of the enzyme. This idea will be developed further in the Discussion section.

Sedimentation Coefficient.—The sedimentation coefficient of the lysin was estimated by sucrose gradient centrifugation (27) as described in Materials and Methods. By plotting the published $S_{20,w}$ values of the reference proteins (soybean trypsin inhibitor, ovalbumin, and bovine serum albumin) (31) *versus* the distance of migration from the meniscus, it was found that the migration

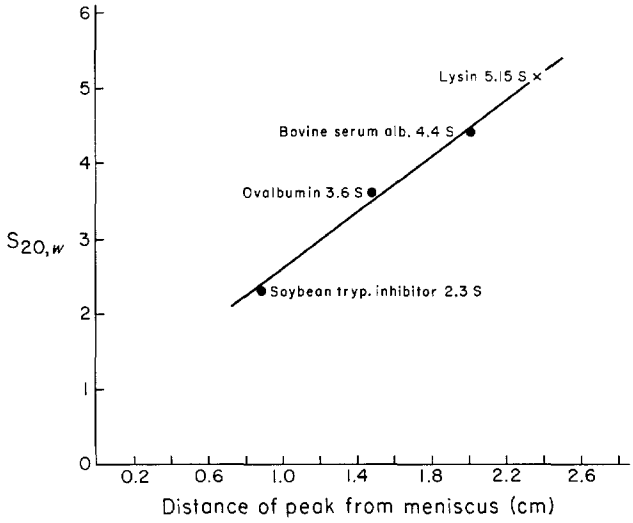

FIG. 4. Plot of the sedimentation coefficient ($S_{20,w}$) of the reference proteins against their peak distance of migration from the meniscus in a 5–20% sucrose gradient. The peak distance of migration of the lysin (purified, step 4) corresponds to an $S_{20,w}$ value of 5.15 on the reference line.

of the lysin corresponds to an $S_{20,w}$ value of 5.2. This value is an average of 5.15 S (Fig. 4) and 5.25 S obtained on another run.

Molecular Weight and Frictional Ratio of Lysin.—The molecular weight (M) and frictional ratio (f/f_0) of the lysin were calculated by the following equations (29):

$$M = 6\pi\eta Nas/(1 - \bar{v}\rho) \quad \{1\}$$

$$f/f_0 = a \Big/ \left(\frac{3\bar{v}M}{4\pi N}\right)^{1/3} \quad \{2\}$$

Where a = Stokes radius, s = sedimentation coefficient, $\bar{v}$ = partial specific volume, η = viscosity of medium, ρ = density of medium, N = Avogadro's

number. Before these formulas can be applied, the Stokes radius of the lysin must be computed (28, 29).

By utilizing the K_d values obtained for the reference proteins (bovine serum albumin, ovalbumin, soybean trypsin inhibitor) by Sephadex G-100 chromatography (see above), along with the calculated Stokes radii for these proteins,[2] the Sephadex G-100 column can be calibrated (by the method of Ackers (28)) to estimate the Stokes radius of the phage lysin. The value for the phage lysin by this method was 46.9 A. Using this figure for the Stokes radius in formulas {1} and {2}, along with an assumed partial specific volume ($\bar{v}$) of 0.725 (32), and sedimentation value for the lysin of 5.2 S (see above), the molecular weight of the enzyme was calculated to be 101,000 with a frictional ratio of 1.526.

DISCUSSION

Despite the widespread use of the Group C phage-associated lysin as a tool in the isolation and study of various streptococcal components, relatively few attempts had been made to obtain a purified preparation for definitive studies concerning its action on the streptococcal cell wall. In all probability the reasons for this relative lack of interest in purifying the enzyme can be linked to its marked instability, as well as to the low yields (approximately 3 mg of purified enzyme/10 liters of phage-infected streptococci).

Previous attempts to purify the Group C phage-associated lysin relied on the constant presence of reducing agents, as well as EDTA to protect the enzyme's labile -SH group(s) from thiol-inactivating agents (13, 33). The stability of the enzyme under these conditions during the purification process or storage at −51°C was found to be quite variable in our hands. It was also evident that the reducing agents interfered with many of the standard methods for estimating protein concentration. By treating the phage lysin with sodium tetrathionate, the enzyme's essential thiol group(s) are converted to the sulfenyl thiosulfate form (Protein-S-S-SO_3^-), thereby protecting them from thiol-inactivating agents (34–37). However, upon addition of reducing agents (DTT) the original activity could be fully regenerated. This was found to be a more specific and effective method for dealing with the problem of instability. In a similar manner, Doughty and Mann (18) utilized *p*-hydroxymercuribenzoate to protect the -SH groups of a staphylococcal phage-induced enzyme. With the -SH groups blocked with tetrathionate, the streptococcal phage enzyme remained

[2] Stokes radii were calculated by the formula (29):

$$a = kT/6\pi\eta D$$

Where a = Stokes radius, T = absolute temperature, k = Boltzman Constant, η = viscosity of the medium, D = diffusion coefficient (as reported in Sober [31]).

extremely stable at 4°C during the purification procedure or during storage at −51°C, provided 0.005 M EDTA and 10% glycerol were present in the buffer. The stabilizing effect of glycerol was found to be particularly important during the final steps in the purification procedure and during storage.

The purification procedure described is the most rapid and least cumbersome to date for obtaining purified phage lysin free of the contaminating Group C carbohydrate (5, 13, 33). The reproducibility of the procedure is excellent and yields from 25 to 34% have been consistently achieved in several experiments. Purification steps monitored by polyacrylamide gel electrophoresis have indicated that the enzyme activity was restricted to a single protein band. A polyacrylamide gel after the final purification step revealed that the enzyme solution was essentially free of major protein contaminants and that the enzyme-active band accounted for 70% of the total protein. As can be seen from Table I, there was a slight increase in the total number of units after the second purification step. This was consistently seen in all preparations and was probably due to the loss of an inhibitor during cellulose phosphate chromatography. Also, since it had been shown that some lytic enzyme is directly associated with the C1 phage particle (19), the 33% loss which was observed after step 1 could be partially accounted for by the loss of bacteriophage from the enzyme solution after centrifugation at 36,000 rpm.

Using the purified lysin preparation, the physical properties of the enzyme were determined by various methods. The gel filtration data on Sephadex G-100 indicated that the molecular weight of the phage lysin was 105,000. However, it was shown by Siegel and Monty (29) that the elution position of a protein by Sephadex chromatography does not necessarily correlate with its molecular weight but is actually a function of its molecular (Stokes) radius. Since many proteins do not possess globular configurations, a comparison between the elution positions of these proteins with those of standard reference globular proteins introduces certain errors in the molecular weight determination. As can be seen from equation {1}, the true value for the molecular weight is dependent upon the values of three parameters: Stokes radius, sedimentation coefficient, and partial specific volume. The estimation of molecular weight by density gradient centrifugation (27) or gel filtration (26) involves the experimental determination of only a single parameter. The combination of these two experimental techniques allows for a more reliable estimation of molecular weight.

By using the peak elution positions of macromolecules of known Stokes radii on Sephadex G-100, Ackers (28) devised a method by which the column can be calibrated in order that the Stokes radii of unknown macromolecules can be calculated. Using experimental determinations for the Stokes radius and sedimentation coefficient, as well as an assumed $\bar{v}$ of 0.725 (most proteins have partial specific volumes between 0.700 and 0.750 cm^3/g) (32), the molecular

weight of the phage lysin was calculated to be 101,000 with a frictional ratio of 1.526 (equations {1} and {2}).

The gel filtration data presented in Fig. 3 indicate that the K_d value for the crude (step 1) lysin corresponds to a molecular weight value which was beyond the resolution of the G-100 column ($\geq$ 150,000) as compared with a molecular weight estimate of 105,000 for the purified enzyme on the same column. The fact that the Group C carbohydrate, as well as other cell wall degradation products, was present in the crude enzyme preparation during chromatography and not in the purified lysin, could explain this molecular weight difference. The crude phage enzyme preparation probably contains large amounts of substrate fragments able to bind to the enzyme, thus allowing this large complex to elute from the Sephadex G-100 column sooner than the smaller noncomplexed purified enzyme.

Now that a supply of stable purified phage enzyme is more easily available, extensive studies of the streptococcal cell wall and protoplast can be performed without fear of introducing contaminating fractions from the Group C cells. Work is presently in progress to determine the cell wall fractions released by the enzyme, as well as its site of action.

SUMMARY

A purification procedure for the Group C phage-associated lysin is described utilizing tetrathionate to protect the enzyme's -SH group(s) from thiol-inactivating agents. A 652-fold purification has been accomplished yielding a solution in which the enzyme activity corresponds to essentially a single band on polyacrylamide gel which accounts for 70% of the total protein in the preparation. A molecular weight of 101,000 and frictional ratio of 1.526 was determined for the lysin utilizing experimentally determined values for its Stokes radius and sedimentation coefficient.

The authors are grateful to Dr. T. Y. Liu for his invaluable advice in the various aspects of the purification procedure, and to Dr. Macyln McCarty for the many stimulating and informative discussions concerning this work, as well as for the generous use of his laboratory facilities.

This investigation was supported in part by U.S. Public Health Service grants AI-02874 and HE03919, Graduate Training grant GM01290 (V.A.F.), and Research Career award 5K6-AI-14,198 (A.W.B.).

BIBLIOGRAPHY

1. Maxted, W. R. 1957. The active agent in nascent phage lysis of streptococci. *J. Gen. Microbiol.* **16**:584.
2. Krause, R. M. 1957. Studies on bacteriophages of hemolytic streptococci. I. Factors influencing the interaction of phage and susceptible host cell. *J. Exp. Med.* **106**:365.
3. Krause, R. M. 1958. Studies on the bacteriophages of hemolytic streptococci. II.

Antigens released from the streptococcal cell wall by a phage-associated lysin. *J. Exp. Med.* **108**:803.

4. Maxted, W. R., and H. Gooder. 1958. Enzymic lysis of Group A streptococci. *J. Gen. Microbiol.* **18**:xiii.
5. Kantor, F. S., and R. M. Cole. 1960. Preparation and antigenicity of M protein released from Group A, Type 1 streptococcal cell walls by phage-associated lysin. *J. Exp. Med.* **112**:77.
6. Krause, R. M., and M. McCarty. 1961. Studies on the chemical structure of the streptococcal cell wall. I. The identification of a mucopeptide in the cell walls of Groups A and A-variant streptococci. *J. Exp. Med.* **114**:127.
7. Freimer, E. H., R. M. Krause, and M. McCarty. 1959. Studies of L forms and protoplasts of Group A streptococci. I. Isolation, growth, and bacteriophage characteristics. *J. Exp. Med.* **110**:853.
8. Freimer, E. H. 1963. Studies of L forms and protoplasts of Group A streptococci. *J. Exp. Med.* **117**:377.
9. Markowitz, A., and A. Dorfman. 1962. Synthesis of capsular polysaccharide (hyaluronic acid) by protoplast membrane preparations of Group A streptococci. *J. Biol. Chem.* **237**:273.
10. Zeleznick, L. D., J. J. Boltralic, S. S. Barkulis, C. Smith, and H. Heymann. 1963. Biosynthesis of streptococcal cell walls: a rhamnose polysaccharide. *Science* (Washington). **140**:400.
11. Fox, E. N. 1963. Intracellular M-protein of Group A streptococcus. *J. Bacteriol.* **85**:536.
12. Doughty, C. C., and J. A. Hayashi. 1961. Enzymatic properties of a phage-induced lysin affecting Group A streptococci. *J. Bacteriol.* **83**:1058.
13. Barkulis, S. S., J. J. Boltralik, and H. Heymann. 1964. Structure of streptococcal cell walls. IV. Purification and properties of streptococcal phage muralysin. *J. Biol. Chem.* **239**:4027.
14. Ralston, D. J., M. Lieberman, B. Baer, and A. P. Krueger. 1957. Staphylococcal virolysin, a phage-induced lysin; its differentiation from the autolysin of normal cells. *J. Gen. Physiol.* **40**:791.
15. Humphries, J. C. 1948. Enzymic activity of bacteriophage–culture lysates. I. A capsule lysin active against *Klebsiella pneumoniae*, type H. *J. Bacteriol.* **56**:683.
16. Murphy, J. S. 1957. A phage-associated enzyme of *Bacillus megaterium* which destroys the bacterial cell wall. *Virology.* **4**:563.
17. Welker, N. E. 1967. Purification and properties of a thermophilic bacteriophage lytic enzyme. *J. Virol.* **1**:617.
18. Doughty, C. C., and J. A. Mann. 1967. Purification and properties of a bacteriophage-induced cell peptidase from *Staphylococcus aureus*. *J. Bacteriol.* **93**:1089.
19. Fox, E. N., and M. K. Wittner. 1965. Observations on the Group C streptococcal bacteriophage and lytic enzyme system. *J. Bacteriol.* **89**:496.
20. Maass, D., and W. Weidel. 1963. Final proof for the identity of enzymic specificities of egg-white lysozyme and phage T2 enzyme. *Biochim. Biophys. Acta.* **78**:369.
21. Bleiweis, A. S., and L. N. Zimmerman. 1961. Formation of two types of osmotically fragile bodies from *Streptococcus faecalis* Var. liquefaciens. *J. Microbiol.* **7**:363.

22. Reiter, B., and J. D. Oram. 1963. Group N streptococcal phage lysin. *J. Gen. Microbiol.* **32:**29.
23. Zabriskie, J. B., and E. H. Freimer. 1966. An immunological relationship between the Group A streptococcus and mammalian muscle. *J. Exp. Med.* **124:**661.
24. Gilman, A., F. S. Philips, E. S. Koelle, R. P. Allen, and E. St. John. 1946. The metabolic reduction and nephrotoxic action of tetrathionate in relation to a possible interaction with sulfhydryl compounds. *Amer. J. Physiol.* **147:**115.
25. Davis, B. J. 1964. Disc electrophoresis. II. Method and application to human serum proteins. *Ann. N. Y. Acad. Sci.* **121:**404.
26. Andrews, P. 1964. Estimation of the molecular weights of proteins by Sephadex gel filtration. *Biochem. J.* **91:**222.
27. Martin, R. G., and B. N. Ames. 1961. A method for determining the sedimentation behavior of enzymes: application to protein mixtures. *J. Biol. Chem.* **236:** 1372.
28. Ackers, G. K. 1964. Molecular exclusion and restricted diffusion processes in molecular-sieve chromatography. *Biochemistry.* **3:**723.
29. Siegel, L. M., and K. J. Monty. 1966. Determination of molecular weights and frictional ratios of proteins in impure systems by use of gel filtration and density gradient centrifugation. Application to crude preparations of sulfite and hydroxylamine reductases. *Biochim. Biophys. Acta.* **112:**346.
30. Whitaker, R. C. 1963. Determination of molecular weights of proteins by gel filtration on Sephadex. *Anal. Chem.* **35:**1950.
31. Sober, H. A. 1968. Handbook of Biochemistry. The Chemical Rubber Company, Cleveland, Ohio.
32. Edsall, J. T. 1953. *In* The Proteins. H. Neurath and K. Bailey, editors. Academic Press, Inc., New York. **1(B):** 549.
33. Tourville, D. R., and S. Tokuda. 1967. Lactic streptococcal phage-associated lysin. II. Purification and characterization. *J. Dairy Sci.* **50:**1.
34. Liu, T. Y. 1967. Demonstration of the presence of a histidine residue at the active site of streptococcal proteinase. *J. Biol. Chem.* **242:**4029.
35. Pihl, A., and R. Lange. 1962. The interaction of oxidized glutathione, cystamine monosulfoxide, and tetrathionate with the -SH groups of rabbit muscle D-glyceraldehyde 3-phosphate dehydrogenase. *J. Biol. Chem.* **237:**1356.
36. Englund, P. T., T. P. King, L. C. Craig, and A. Walti. 1968. Studies on ficin. I. Its isolation and characterization. *Biochemistry.* **7:**163.
37. Glazer, A. N. 1970. Specific chemical modification of proteins. *Annu. Rev. Biochem* **39:**101.