

The Lysozyme of Bacteriophage λ

I. PURIFICATION AND MOLECULAR WEIGHT*

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SUMMARY

The lysozyme synthesized in *Escherichia coli* during the development of bacteriophage λ (λ -lysozyme) has been purified to homogeneity from either the whole cell just prior to lysis or from the lysate. The molecular weight of this protein was determined from its $s_{20,w}$ (2.06 S) and its $D_{20,w}$ ($10.5 \times 10^{-7} \text{ cm}^2 \cdot \text{sec}^{-1}$) as well as from sedimentation equilibrium data to be 17.9×10^3 . Differences between the catalytic activities of λ -lysozyme and egg white lysozyme on a limited group of substrates have been observed.

It has been known for some time that a lysozyme, or endolysin, appears during the development of bacteriophage λ in *Escherichia coli* K12 (1). This lysozyme is not detected in sensitive *E. coli* previous to infection by λ or in *E. coli* lysogenic for λ previous to their induction. Presumably the synthesis of the lysozyme is necessary for the breakdown of the *E. coli* cell wall that allows the release of the newly synthesized λ phages from the infected bacterium.

Our initial interest in this enzyme was based on two suppositions. By analogy to other lysozymes, particularly that of the coliphage T4 (2), the supposition was made that λ -lysozyme is a simple protein consisting of only one small polypeptide chain. This first supposition is established in this and the two succeeding articles (3, 4). The second supposition was that *R*, the structural gene for λ -lysozyme, is located near one end of the linear duplex DNA isolated from mature λ . This supposition was induced by the location of *R* as the most terminal gene on the right side of the genetic map of vegetative λ (5), and has been established by determining the gene content of fragments of λ DNA which vary in size but have in common the right end of the whole λ DNA (6).

Smallness of the polypeptide is a virtue for the determination of the position and nature of changes in its structure caused by mutation in its gene. Insofar as smallness indicates simplicity, it should also be a useful characteristic in developing a system for

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the synthesis of a polypeptide starting from DNA. In this case, smallness of the DNA molecules containing the required gene may also be a virtue. The location of *R* near the end of λ DNA has allowed the isolation of small DNA fragments which contain *R* and the right-hand terminus of λ DNA, but none of the other λ genes.¹

With these possibilities and associated advantages in mind, we report on the characterization of λ -lysozyme in this set of three articles. In this first article we determine the molecular weight of λ -lysozyme by sedimentation analysis and describe the purification procedures which yield the necessary homogeneous preparations. The experiments which relate to the primary structure of λ -lysozyme are described in the succeeding two articles (3, 4) of this set.

EXPERIMENTAL PROCEDURE

Materials

Bacteria and Phages—The source of λ -lysozyme was *E. coli* K12 (strain W3104 of E. Lederberg) made doubly lysogenic for λ and *ldg* (7) and induced by ultraviolet light. As W3104 lysogenic for λ alone yields lysates with the same specific activity for λ -lysozyme as does the above double lysogen, it would presumably represent as good a source.

Reagents—Hydroxylapatite was Hypatite C from the Clarkson Chemical Company. Carboxymethyl Sephadex C-50 was obtained from Pharmacia. Amberlite XE-64 was a product of Rohm and Haas and was treated by the method of Hirs, Moore, and Stein (8). Streptomycin sulfate was a gift from Merck, Sharp and Dohme. Hydrolyzed starch used for gel electrophoresis was purchased from Connaught Laboratories, Toronto. The reagents used for preparation of polyacrylamide gels were obtained from Eastman Kodak.

Egg white lysozyme (twice crystallized, Batch LY616) was obtained from Worthington and *E. coli* DNA labeled with ³²P was a gift of A. V. Paul.

Methods

Assay of λ -Lysozyme—The activity of the enzyme was assayed by observing its ability to decrease the absorbance at 600 μ caused by the turbidity of chloroform-treated *E. coli* K12 (strain

¹ J. B. Egan and D. S. Hogness, unpublished experiments.

W3104). The bacterial substrate was prepared from cells growing exponentially at 37° in 3 liters of M-9 medium (9) supplemented with 3 mg of glucose per ml. The cells were harvested by centrifugation (0–2°, 15 min, 13,200 × *g*) when their concentration was 4×10^8 cells per ml and then resuspended at 25° in 400 ml of dilution solvent (0.005 M EDTA, 0.06 M potassium phosphate buffer, pH 7.0) saturated with chloroform. Immediately upon completing the suspension, it was chilled to 0°, and the cells were washed at 0–2° by three cycles of centrifugation and resuspension in dilution solvent, with 200 ml in the first two cycles and 50 ml in the last. Samples were frozen in liquid nitrogen and stored at –20°. No loss of sensitivity in the assay was detected after storing for 6 months, but repeated thawing and freezing of the same sample should be avoided as this significantly decreases the sensitivity.

After diluting the enzyme in the dilution solvent, 0.05 ml is added to one of two tubes, each of which contains 1.0 ml of thawed bacteria diluted in the same solvent to an absorbance at 600 *mμ* of 0.5. Immediately thereafter 0.05 ml of dilution solvent is added to the other tube and the two tubes are placed in a water bath at 37° for 4.0 min. The contents are then transferred to a cuvette, and the absorbance is determined at 4.5 min. The difference in absorbance between the two suspensions (Δ absorbance) is proportional to enzyme concentration as long as the Δ absorbance does not exceed 0.3. One unit of λ -lysozyme is defined as that amount which will cause a Δ absorbance of 0.1. The following compounds used in the purification of the enzyme inhibit its activity and should be removed prior to assay: $(\text{NH}_4)_2\text{SO}_4$, streptomycin sulfate, and protamine sulfate.

The assay was standardized with respect to different batches of bacterial substrate by testing their sensitivity to egg white lysozyme which had a specific activity within 10% of 5000 λ -lysozyme units per mg for all batches of substrate.

Other Assays and Measurements—The procedure for the phage assay has been described (7), as has that for determining the activity of λ -exonuclease (10).

Protein was measured by the method of Lowry *et al.* (11), with crystalline bovine serum albumin as a standard. The protein concentration of purified λ -lysozyme calculated with this standard is evidently accurate since the refractive index increment determined according to Van Holde and Baldwin (12) using protein concentrations determined in this manner is 1.86×10^{-4} ml per mg, in agreement with the values found for other simple proteins ($1.86 \pm 0.02 \times 10^{-4}$ ml per mg (13)).

In some cases, the protein concentration of purified λ -lysozyme was determined from the absorbance at 280 *mμ* and the value for its extinction coefficient which we observed to be 1.07 *cm*² per mg.

Absorbance was measured in a Zeiss PMQII spectrophotometer with a 1-cm light path.

Analytical sedimentations were performed in a Spinco model E ultracentrifuge with schlieren optics and Eastman Spectroscopic II G plates to photograph the patterns, which were measured with a Gaertner M2001RS two-dimensional microcomparator.

Purification of λ -Lysozyme

Induction of Lysogenic Cells—The kinetics of appearance of λ -lysozyme, λ -exonuclease, and newly formed λ phages following induction of lysogenic bacteria with ultraviolet light is given in Fig. 1. They indicate the “late” nature of *R*, the structural gene for λ -lysozyme, as compared to the “early” characteristic of the gene for λ -exonuclease, this same temporal sequence hav-

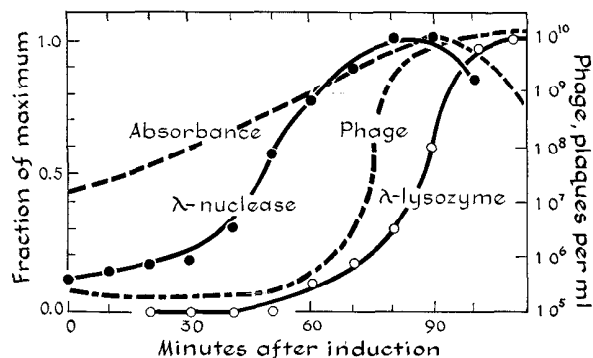


FIG. 1. Kinetics of appearance of λ -lysozyme after induction with ultraviolet light. W3104 lysogenic for λ were grown exponentially in 200 ml of H medium (14) containing 4 mg of glucose per ml to a bacterial density of 7×10^8 bacteria per ml. After chilling to 0°, the suspension was irradiated for 70 sec with ultraviolet light at a surface intensity of 19 ergs *mm*^{–2} *sec*^{–1} in a tray, 22 × 33 cm, under mild agitation. Twenty milliliters of 10% Difco Bactotryptone were added, the culture was returned to 37° at zero time on the above abscissa, and the growth was followed by measuring the absorbance at 600 *mμ*. Samples were removed at various times, chilled to 0°, and assayed for the indicated characteristics. Total infective phage were assayed after treating a 2-ml sample with 2 drops of chloroform plus 0.02 ml of a 5 mg per ml solution of egg white lysozyme in 0.1 M potassium EDTA, pH 7.0, shaking vigorously, and incubating at 37° for 20 min. Extracts for enzyme assays were made by sonic oscillation (Mullard Sonicator, 0°, 60 sec) of bacterial suspensions prepared by centrifuging the sample and resuspending the pellet in an equal volume of 0.05 M potassium glycyl-glycine buffer, pH 7.0. Note that the phage yield is plotted logarithmically and consequently gives the impression of premature appearance in comparison with the other quantities; *e.g.* the time at which the phage yield attains 5% of its maximum value is later than the comparable time for λ -lysozyme, although the logarithmic phage curve appears to precede the λ -lysozyme curve.

ing been observed previously when lysogenic cells are induced with mitomycin (15) or by thymine starvation (16) and when sensitive cells are infected with λ (16). Clearly, the best source of λ -lysozyme is either the cells just prior to lysis or the lysate itself. Both sources have been used. As the lysate is the preferred source for the isolation of large amounts of the wild type enzyme, we have described the purification from this source in some detail. The purification of the enzyme from cells prior to lysis differs appreciably only in the initial steps. These steps are described subsequently since such cells may provide the best source for less stable forms of λ -lysozyme, *e.g.* those derived from temperature-sensitive mutants. The growth and induction of the lysogenic bacteria given below are the same for each procedure.

E. coli K12 doubly lysogenic for λ and λ dg (7) were grown overnight at 37° in a Biogen (American Sterilizer Corporation) containing 90 liters of H medium (14) supplemented with 81 g of glucose which limits the overnight growth to 7×10^8 cells per ml. After adding 320 g of glucose the next morning to resume growth, cooling of the culture to 26° was initiated when the cell density was 1.5×10^9 cells per ml and completed when it had risen to 2×10^9 cells per ml. The bacteria were then irradiated for 400 sec with the ultraviolet lamps of the Biogen. Nine liters of 10% Difco Bactotryptone were added along with a small amount of antifoam A (Dow Corning Corporation) and the temperature was adjusted to 37°.

For purification from unlysed cells, the bacteria were allowed

to grow for 55 min after reaching 37°, and then collected rapidly in iced vats and harvested with a Spinco model 170 continuous flow centrifuge at 29,000 $\times g$, keeping the temperature below 15°. The centrifuged bacteria were quickly frozen in liquid nitrogen and stored at -20°.

For purification from lysates the culture was maintained at 37° until completion of lysis and then cooled to 0°. Under these conditions of growth and induction, lysis normally begins at 60 min and is complete by 130 min.

The details of the procedure for the purification of λ -lysozyme from this lysate are given below and the results are given in Table I. When the total volume listed in Table I is greater than that given below, it indicates that multiple batches were used. Unless otherwise stated, all manipulations in the following procedures were performed at 0-5° and all centrifugations were at 13,200 $\times g$ for 15 min.

Precipitation at pH 3.2—HCl (250 ml) 2 N was slowly stirred into 30 liters of lysate until the pH reached 3.2. After 10 min of further stirring, the precipitate was centrifuged from suspension at 29,000 $\times g$ and 10-15° with a Spinco model 170 continuous flow centrifuge, and then resuspended in 3 liters of 0.005 M EDTA, 0.08 M potassium phosphate buffer, pH 6.4, by stirring at top speed in a Waring Blender for 2 min. This suspension was stirred overnight and centrifuged, the supernatant was saved, and the pellet was resuspended in 170 ml of 0.005 M EDTA, 0.08 M potassium phosphate buffer, pH 6.4. After centrifugation of this second suspension, the two supernatants were combined to yield Fraction II.

Streptomycin Fractionation—To 3 liters of Fraction II was added, with stirring, 0.03 times its volume of 25% streptomycin sulfate dissolved in 0.005 M EDTA, 0.08 M potassium phosphate buffer, pH 6.4. The mixture was stirred slowly for 15 min and

centrifuged, and the supernatant was collected to obtain Fraction III.

Ammonium Sulfate Fractionation—To 3 liters of Fraction III were added, with stirring, 730 g of solid $(\text{NH}_4)_2\text{SO}_4$. After 20 min of further stirring, the suspension was centrifuged and 680 g of solid $(\text{NH}_4)_2\text{SO}_4$ were added to the resulting supernatant in the same manner. The precipitate obtained by centrifugation was dissolved in 250 ml of 0.005 M EDTA, 0.08 M potassium phosphate buffer, pH 6.4, to obtain Fraction IV. Four batches of Fraction IV were combined and dialyzed against 6 liters of the preceding buffer, the dialysate being changed once during an overnight period.

Amberlite XE-64 Chromatography—A column of Amberlite XE-64 (3.3 cm \times 26 cm) was washed with 4 column volumes of 0.005 M EDTA, 0.08 M potassium phosphate buffer, pH 6.4. After centrifugation of Fraction IV to remove the fine precipitate which formed during dialysis, it was applied to the column at a flow rate of 2.2 ml per min. The column was washed with 1 column volume of 0.005 M EDTA, 0.1 M potassium phosphate buffer, pH 6.4, and a constant elution gradient from zero to 0.7 M KCl in 2 liters of the preceding solvent was applied at 2.2 ml per min, 10-ml fractions being collected. The enzyme was eluted in the second quarter of the gradient and pooled to form Fraction V.

Acetone Precipitation—Acetone was added slowly with stirring to Fraction V (585 ml) until a final acetone concentration of 70% (v/v) was achieved. After standing for 30 min, the resulting suspension was centrifuged for 15 min at 5860 $\times g$ and the precipitate was dissolved in 140 ml of 0.001 M EDTA, 0.05 M potassium phosphate buffer, pH 6.0. This solution was dialyzed against 6 liters of the same solvent, the dialysate being changed once during the overnight dialysis (Fraction VI).

Carboxymethyl Sephadex C-50 Chromatography—A column of carboxymethyl Sephadex C-50 (2.2 cm \times 10.5 cm) was washed with 2 column volumes of 0.001 M EDTA, 0.05 M potassium phosphate, 0.4 M KCl buffer, pH 6.0, and then with 10 column volumes of 0.001 M EDTA, 0.05 M potassium phosphate buffer, pH 6.0. Following centrifugation, the dialyzed Fraction V (150 ml) was applied at 1 ml per min, the column was then washed with 4 column volumes of 0.001 M EDTA, 0.1 M KCl, 0.05 M potassium phosphate buffer, pH 6.0. A constant gradient from 0.10 M KCl to 0.30 M KCl in 500 ml of 0.001 M EDTA, 0.05 M potassium phosphate buffer, pH 6.0, was then applied at a flow rate of 1 ml per min and 6.5-ml fractions collected. The fractions containing enzyme were pooled and dialyzed against 6 liters of 0.005 M potassium phosphate buffer, pH 6.0, to obtain Fraction VII.

Hydroxylapatite Chromatography—A column of hydroxylapatite (2.2 cm by 5.3 cm) was washed with 2 column volumes of 0.225 M potassium phosphate buffer, pH 6.8, and then with 3 column volumes of 0.005 M potassium phosphate buffer, pH 6.8. Fraction VII (155 ml) was loaded on the column at a flow rate of 0.5 ml per min and eluted at the same flow rate by a constant gradient between 0.025 M potassium phosphate buffer, pH 6.8, and 0.225 M potassium phosphate buffer, pH 6.8, in a total volume of 400 ml. Fractions of 7.5 ml were collected and Fractions 21 to 37 (Fig. 2) were pooled to form Fraction VIII of Table I. This lysozyme was concentrated by acetone precipitation to varying degrees depending upon its usage. Such concentrated solutions are also referred to as Fraction VIII in the succeeding sections.

TABLE I
Purification of λ -lysozyme

| Fraction and step | Volume liters | Protein | | λ -Lysozyme | | |
|---|------------------|------------|------------------------|------------------------------------|---|---|
| | | Total g | Concentration mg/ml | Total units $\times 10^{-6}$ | Concentration units/ml $\times 10^{-3}$ | Specific activity units/mg $\times 10^{-3}$ |
| I. Lysate | 120 | | | 20 | 0.17 | |
| II. Precipitation at pH 3.2 | 11.5 | 34 | 3.0 | 5.9 | 0.51 | 0.17 |
| III. Streptomycin fractionation | 11.7 | 32 | 2.7 | 5.6 | 0.48 | 0.18 |
| IV. $(\text{NH}_4)_2\text{SO}_4$ fractionation | 1.20 | 10.2 | 8.5 | 5.6 | 4.7 | 0.55 |
| V. Amberlite XE- 64 chroma- tography | 0.58 | 0.29 | 0.50 | 5.6 | 9.6 | 19 |
| VI. Acetone pre- cipitation | 0.150 | 0.180 | 1.20 | 5.2 | 35 | 29 |
| VII. Carboxy- methyl Sephadex C-50 chro- matography | 0.155 | 0.115 | 0.74 | 5.1 | 33 | 45 |
| VIII. Hydroxylapa- tite chroma- tography | 0.125 | 0.090 | 0.72 | 4.5 | 36 | 50 |

Purification from Unlysed Cells—The preparation of a crude extract from the frozen bacteria and its fractionation with streptomycin are given below. Final purification was achieved by procedures essentially the same as those given above, starting with the ammonium sulfate fractionation.

The frozen bacteria (650 g, wet weight) were partially thawed and suspended in 2600 ml of acetone in a Waring Blendor for 15 min at medium speed. The acetone was removed by filtration and the bacteria were thoroughly dried. The enzyme was stable in the acetone powder for at least 6 months at -20° . Fifty grams of acetone powder were suspended in 750 ml of 0.005 M EDTA, 0.08 M potassium phosphate buffer, pH 6.4, by stirring overnight. The suspension was centrifuged, the pellet was re-suspended in 750 ml of the preceding solvent, and this second suspension was centrifuged. After combining the two supernatants, the protein concentration was adjusted to 10 mg per ml by dilution with the above buffer, and 1 volume of 25% streptomycin sulfate (dissolved in the same buffer) was added, with stirring, to every 10 volumes of the extract. The mixture was stirred for 15 min and centrifuged, and the supernatant (2130 ml) was collected and fractionated with ammonium sulfate as described above.

Enzyme prepared from each of the two sources (specific activity of 45 to 50×10^3 units per mg of protein) has been used for characterization of the sedimentation coefficient and the molecular weight. No significant differences were detected, indicating that the low pH used to precipitate protein from the lysate has little, if any, irreversible effect.

RESULTS

Tests for Homogeneity

Fig. 2 shows the chromatography of Fraction VII on a hydroxylapatite column, the last step in the purification procedure. The single peak of λ -lysozyme activity coincides with the single protein peak to the extent that the specific activities are within $\pm 15\%$ of a single value (50×10^3 units per mg), which is within the combined error of the assays.

The results of electrophoresis at pH 5 in starch gels of both a purified preparation and one in which about one-half of the protein was λ -lysozyme are shown in Fig. 3. That the strongly

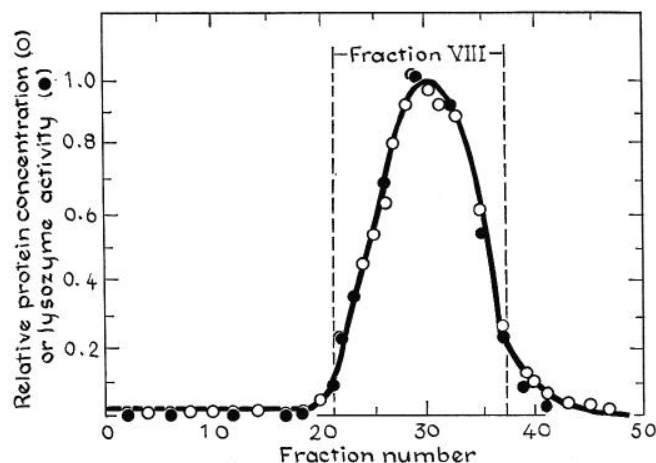


Fig. 2. Hydroxylapatite chromatography of Fraction VII. The conditions are given in the text. The maximum protein concentration and lysozyme activity, found in Fraction 29, were 1.5 mg per ml and 76×10^3 units per ml, respectively.

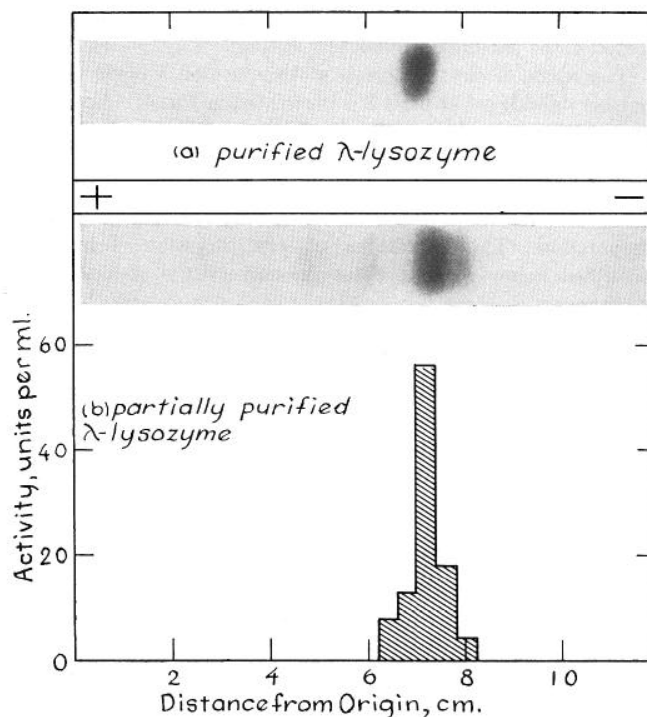


Fig. 3. Starch gel electrophoresis of λ -lysozyme. The purified enzyme was used in *a* while *b* represents the result with a partially purified preparation whose specific activity is one-half the maximum value. Gels were made in 0.05 M sodium acetate buffer, pH 5.0, according to the method of Smithies (17). The slot was loaded with 0.05 ml of enzyme solution (3 mg per ml for Fraction VI and 5 mg per ml for Fraction VIII), and 175 volts were applied for 14 hours across electrodes connected to reservoirs containing 0.05 M sodium citrate buffer, pH 5.0. The gels were sectioned and stained according to Wilson and Hogness (18). The lower curve represents the λ -lysozyme activity eluted from 0.5-cm sections of the unstained half of the gel into 1 ml of 0.06 M potassium phosphate buffer, pH 7.0, after a 14-hour exposure at $2-4^{\circ}$.

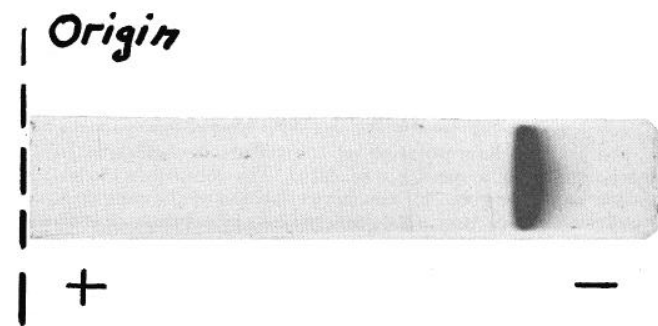


Fig. 4. Polyacrylamide gel electrophoresis of λ -lysozyme. The electrophoresis of Fraction VIII was performed according to the method of Ornstein and Davis (19). A 15% small pore gel stacked at pH 5 and run at pH 4.3 was used (see Reference 20 for the composition of gels and buffers). Following electrophoresis of $75 \mu\text{g}$ of λ -lysozyme for 1 hour (voltage drop, 150 volts; current, 2.5 ma; $2-4^{\circ}$), the gel was stained with Amido black and washed with 7% acetic acid.

staining band is the λ -lysozyme is made clear by the measurement of enzyme activities of the material in segments of the gel (Fig. 3b). Starch gel electrophoresis was also performed at pH 7.0, but resolution of impurities was greatly reduced at this pH;

only one band, moving as positively charged protein, was detected in the partially purified preparation.

The result of electrophoresis of the purified λ -lysozyme in a polyacrylamide gel at pH 4.3 is illustrated in Fig. 4. Again only one component is detectable.

The schlieren pattern obtained during the centrifugation of Fraction VIII consisted of a single symmetrical peak indicating the existence of only one sedimenting component in the purified preparation. The conditions of centrifugation were those described below for the determination of the sedimentation coefficient.

Sedimentation Behavior and Determination of Molecular Weight

The previous data indicate that the purified λ -lysozyme is sufficiently homogeneous to allow a determination of its molecular weight by sedimentation analysis. In the following sections, the molecular weight has been determined from the sedimentation and diffusion coefficients and from the radial distribution of the protein after centrifugation to equilibrium.

Sedimentation and Diffusion Coefficients—The sedimentation coefficient ($s_{20,w}$) of λ -lysozyme, measured at a protein concentration of 10.0 mg per ml, is 2.02 S, and at 4.2 mg per ml it is 2.10 S. For these determinations the enzyme, dissolved in 0.10 M KCl, 0.01 M potassium phosphate buffer, pH 7.0, was centrifuged at 59,780 rpm and 23.5° in a 12-mm, 2°, double sector synthetic boundary-forming cell. Although there appears

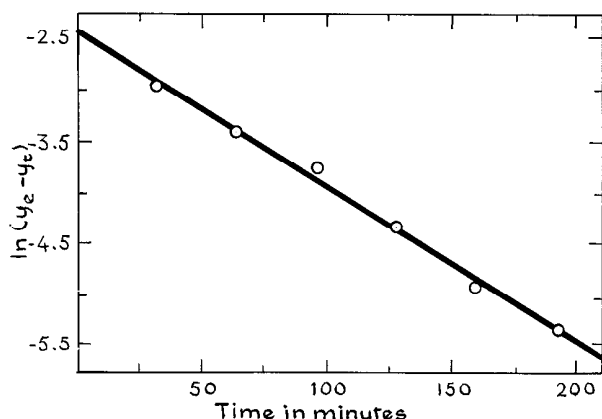


FIG. 5. The determination of the diffusion coefficient by the method of Sophianopoulos *et al.* (21). The conditions of centrifugation are those given for the determination of the sedimentation coefficient except that a 2.04-mm column of solution in a 12-mm, 2° double sector Epon cell was centrifuged at 15,220 rpm, for 25 hours at 18.3°. The schlieren patterns during the approach to equilibrium as well as at equilibrium were photographed and the vertical displacement from the base line at $(b + m)/2$ was measured, where b and m refer to the radial distances to the bottom and meniscus of the solution column, respectively. At equilibrium this vertical displacement is referred to as y_e , while at other times, t , it is referred to as y_t . The diffusion coefficient can then be calculated from the equation (21):

$$D = \frac{(b - m)^2}{\pi^2} \cdot \frac{d \ln (y_e - y_t)}{dt} \left/ \left[1 + \frac{(b - m)^2}{\pi^2} \cdot \left(\frac{\omega^2 M (1 - \bar{v} \rho)}{2RT} \right) \left(\frac{b + m}{2} \right)^2 \right] \right. \cdot \left[1 + \frac{3}{\pi^2} \left(\frac{b - m}{b + m} \right)^2 \right]$$

since the slope of the above curve is equal to $d \ln (y_e - y_t)/dt$. The terms in brackets are small correction terms having a value of 1.052 under the conditions used here.

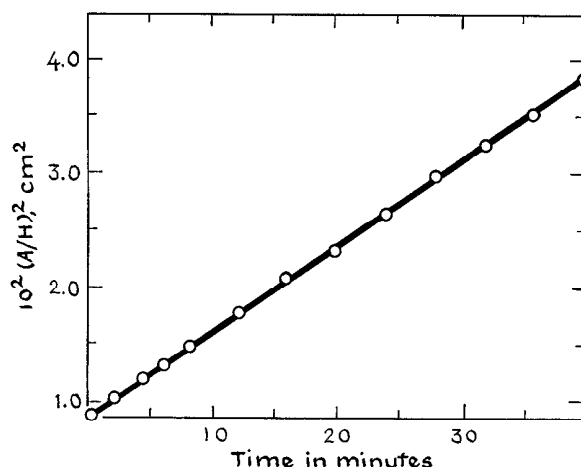


FIG. 6. Determination of the diffusion coefficient of λ -lysozyme from boundary spreading. The conditions of centrifugation were those given for determination of the sedimentation coefficient except that the speed was 8000 rpm and the temperature was 18.3°. The height (h) and area (a) of the single boundary represented in the schlieren photographs were determined as a function of time and the ratio of the area (A) to height (H) of the true boundary curve ($\partial c/\partial r$ against r) calculated from $A/H = a/(h \cdot F)$ where F is the magnification factor for r . D is computed from the slope of the above curve and the equation (22):

$$(A/H)^2 = 4\pi Dt$$

to be some dependence of the $s_{20,w}$ on concentration (the above values extrapolated to zero concentration by plotting either s or s^{-1} versus c give a value of 2.16 at $c = 0$) the data are insufficient for an accurate determination of this dependence. Consequently, we use the average value of 2.06 S for the concentration range 4 to 10 mg per ml.

The diffusion coefficient ($D_{20,w}$) of λ -lysozyme was measured by two different methods at a protein concentration of 10 mg per ml. The method of Sophianopoulos *et al.* (21) was used in analyzing the transient states during sedimentation to equilibrium. The method and data are summarized in Fig. 5, from which a value of 10.7×10^{-7} cm²·sec⁻¹ for the $D_{20,w}$ was calculated.

In the second method, the diffusion coefficient was calculated by measuring the spreading of the boundary formed at 8000 rpm in a synthetic boundary cell as a function of time (22). The data and method are summarized in Fig. 6, from which a value for the $D_{20,w}$ of 10.2×10^{-7} cm²·sec⁻¹ was calculated. The small sedimentation coefficient of λ -lysozyme, the low speed of centrifugation, and the time interval used (36 min) are such that the radial position of the maximum in the schlieren pattern (r_{max}) increases only slightly during measurement of boundary spreading (Δr_{max} at 36 min is about 1% the increment in the width of the schlieren peak at half of the maximum height). Consequently, corrections for sharpening of the boundary due to a dependence of s on concentration can be neglected.

The average value of the diffusion coefficient calculated by the two methods in 10.5×10^{-7} cm²·sec⁻¹ and, when this is combined with the previously determined sedimentation coefficient, a molecular weight for λ -lysozyme of 1.78×10^4 was calculated from the Svedberg equation,

$$M = \frac{sRT}{D(1 - \bar{v} \rho)}$$

The partial specific volume, \bar{v} , was computed from the amino acid composition (3) to be $0.732 \text{ cm}^3 \text{ per g}$ (23).

Sedimentation Equilibrium—Centrifugation of the enzyme to sedimentation equilibrium in a short column of solution (3.52 mm) at 33,450 rpm yields a distribution (Fig. 7) in which the protein concentration at the meniscus is zero. This distribution allows the calculation of the molecular weight from a single schlieren pattern by two methods: (a) from a determination of $d(\ln c_r)/d(r)^2$, where r is the distance from the axis of rotation and c_r is the protein concentration at r (Fig. 7a), and (b) from a determination of $d((1/r) \cdot (dc_r/dr))/dc_r$ (Fig. 7b). The values calculated by these two methods are in good agreement, being 1.75×10^4 and 1.79×10^4 , respectively (see Table II). A disadvantage of the distribution obtained at this relatively high speed is that only a fraction of the total protein added to the centrifuge cell is analyzed (the remainder being in a region of too

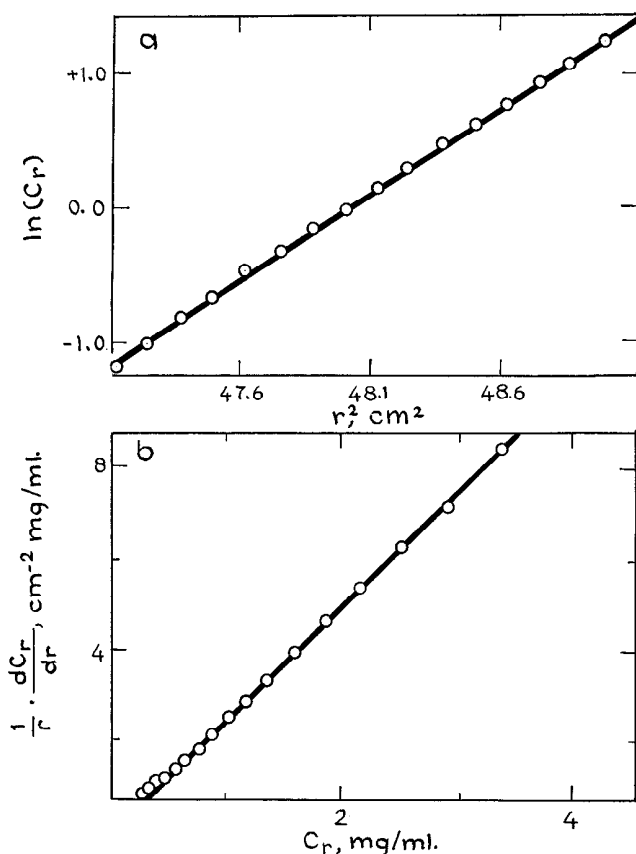


FIG. 7. Determination of the molecular weight of λ -lysozyme by sedimentation to equilibrium. Except for the speed (33,450 rpm) and temperature (16°) the conditions of centrifugation were identical with those given in Fig. 5. The schlieren pattern of the equilibrium distribution was photographed and analyzed to give the plots a and b above. a, the molecular weight, M , is calculated from the equation (24):

$$M = \frac{2RT}{(1 - \bar{v}\rho)\omega^2} \cdot \frac{d(\ln c_r)}{d(r^2)}$$

b, the molecular weight is calculated from the equation (12):

$$M = \frac{RT}{(1 - \bar{v}\rho)\omega^2} \cdot \frac{d\left(\frac{1}{r} \cdot \frac{dc_r}{dr}\right)}{dc_r}$$

TABLE II
Molecular weight of λ -lysozyme

| Method | Angular velocity | pH ^a | c_0^b | Molecular weight |
|--|------------------|-----------------|---------|------------------|
| | rpm | | mg/ml | $\times 10^{-4}$ |
| Equilibrium centrifugation Method a (see Fig. 7a) | 33,450 | 7.0 | 4.2 | 1.75 |
| | 35,600 | 4.9 | 5.0 | 1.82 |
| Method b (see Fig. 7b) | 33,450 | 7.0 | 4.2 | 1.79 |
| | 35,600 | 4.9 | 5.0 | 1.77 |
| | 16,400 | 7.0 | 4.2 | 1.82 |
| | 15,220 | 9.3 | 5.0 | 1.83 |
| Sedimentation-diffusion ^c | | 7.0 | | 1.78 |
| Average | | | | 1.79 |

^a The solvent at pH 7.0 was that given in Fig. 5. At pH 4.9, the solvent was 0.10 M KCl, 0.025 M potassium acetate buffer. At pH 9.3, the solvent was 0.20 M KCl, 0.020 M potassium carbonate buffer.

^b c_0 stands for the initial protein concentration in the equilibrium determinations.

^c $s_{20,w} = 2.06 \text{ S}$; $D_{20,w} = 10.5 \times 10^{-7} \text{ cm}^2 \cdot \text{sec}^{-1}$.

high a dc/dr value to be registered in the schlieren pattern). Consequently, the values of molecular weight obtained by Methods a and b do not necessarily represent the weight average (M_w) and z-average (M_z) molecular weight, respectively, that would otherwise prevail.

At the lower speed of 16,200 rpm, the equilibrium concentration at the meniscus is not zero and must be evaluated for the first of the above methods, although not for the second. Since this evaluation introduces an error in the first method not inherent in the second, we present here only that molecular weight determined by the second method at this lower speed. This value is 1.82×10^4 and compares favorably with the value of 1.79×10^4 calculated by the same method at the higher speed (Table II). Since essentially all of the column of solution is analyzed at the lower speed and consequently most of the protein molecules contribute to the distribution, the agreement between the values calculated at the two speeds indicates that the disadvantage associated with the higher speed measurements is not effective for these preparations. The agreement between the three values is also indicative of the homogeneity of the material.

Effect of pH on Molecular Weight—Sophianopoulos and Van Holde (25) have shown that egg white lysozyme undergoes a reversible dimerization between pH 5 and pH 9, the extent of dimerization increasing with increasing pH. As a consequence, the molecular weight calculated from sedimentation equilibrium measurements is a function of pH and represents the monomer only below pH 5. This type of aggregation does not occur with λ -lysozyme. The molecular weight of λ -lysozyme was determined at pH 4.9 and pH 9.3 by sedimentation to equilibrium. Both Methods a and b were used at the lower pH, while Method b alone was used at the higher pH. It is clear from an examination of the values for molecular weight given in Table II that there is no significant change as the pH is raised from 4.9 to 9.3. All values fall into the range $(1.79 \pm 0.04) \times 10^4$.

Catalytic Properties

While our present interests emphasize the structural characteristics of λ -lysozyme without special reference to its catalytic

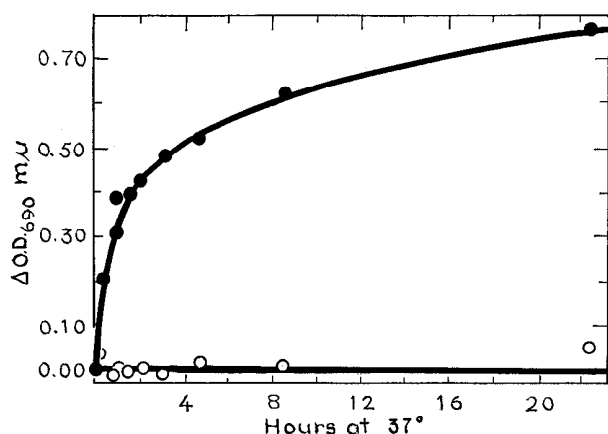


FIG. 8. Action of λ -lysozyme and egg white lysozyme on penta-*N*-acetylglucosamine. Two tubes containing 38 mM sodium citrate, 3.5 mM penta-*N*-acetylglucosamine, pH 5.1, in a final volume of 0.54 ml were incubated at 37° for 24 hours. One tube contained 0.146 mM egg white lysozyme, the other 0.117 mM λ -lysozyme. Samples, 0.025 ml, were removed from the sealed tubes during the incubation, diluted to a final volume of 1 ml, and quickly frozen. Of this solution 0.50 ml was assayed for reducing sugar groups (29). In this assay, the optical density at 690 m μ increases as the concentration of reducing groups increases: ○, λ -lysozyme; ●, egg white lysozyme.

properties, we have made certain observations pertinent to its catalytic function, particularly in comparison to that of egg white lysozyme.

The purified λ -lysozyme has a specific activity 10 times that of egg white lysozyme in our assay system with the *E. coli* substrate. Because the molecular weight of λ -lysozyme is slightly larger than for egg white lysozyme ($M = 14.3 \times 10^3$), the ratio is slightly greater on a units/molecule basis. In agreement with results obtained with impure preparations of λ -lysozyme (26, 27), we find that the purified λ -lysozyme has no effect on *Micrococcus lysodeikticus*; the rate catalyzed by λ -lysozyme was less than 0.001 the rate observed for egg white lysozyme.

We have also investigated the effect of λ -lysozyme on two of the simpler substrates for egg white lysozyme, the tri- and penta-*N*-acetylglucosamine derived from chitin and kindly sent to us by J. A. Rupley (28). Penta-*N*-acetylglucosamine is hydrolyzed by egg white lysozyme at a much greater rate than is tri-*N*-acetylglucosamine (28). However, the penta-*N*-acetylglucosamine is not hydrolyzed by λ -lysozyme (Fig. 8). As the λ -lysozyme was fully active on the *E. coli* substrate after the 22-hour incubation shown in Fig. 8, we conclude that the penta-*N*-acetylglucosamine is not a substrate for this enzyme. Similarly, tri-*N*-acetylglucosamine was observed to be insensitive to attack by λ -lysozyme.

DISCUSSION

From our viewpoint, the most important conclusions of this paper are (a) that the λ -lysozyme is a small protein, (b) that the number of molecules synthesized per induced cells is comparatively high, and (c) that respectable amounts of the pure protein can be isolated in a reasonably simple manner.

The low molecular weight is the result that we had anticipated by analogy to other lysozymes. The value of 17.9×10^3 obtained for λ -lysozyme is remarkably similar to the value of $18.1 \times$

10^3 recently found for the lysozyme derived from coliphage T4 (2) and about 25% greater than the molecular weight of egg white lysozyme. The similarity between the lysozymes of the coliphages and the difference between them and egg white lysozyme is further emphasized by the comparison of their amino acid compositions given in the following paper (3).

The λ -lysozyme and the egg white lysozyme also differ in their catalytic properties. However, the failure of λ -lysozyme to catalyze the hydrolysis of penta-*N*-acetylglucosamine does not provide a very strong argument that with cell wall substrates the two enzymes do not effect the hydrolysis of the same bond, namely the β -(1,4) linkage between the *N*-acetylmuramic acid and *N*-acetylglucosamine residues, the bond attacked by egg white lysozyme (30). Indeed, it is quite possible that λ -lysozyme, but not egg white lysozyme, demands the presence of a muramic acid residue for catalysis. Previous work with impure preparations of λ -lysozyme and cell wall substrates indicates that similar, perhaps identical, products were obtained when λ -lysozyme and egg white lysozyme attacked the cell walls of *Bacillus megaterium*; on the other hand, some dissimilar products were obtained from cell walls of *E. coli* (26). Considering that an impure enzyme was used, these investigations also leave open the question as to what bond in the cell wall is destroyed by λ -lysozyme.

With the molecular weight and the specific activity of the purified enzyme, one can compute the number of λ -lysozyme molecules per induced cell from the enzyme activity in lysates. Under our conditions of induction this turns out to be about 6×10^4 molecules. It is difficult to estimate the efficiency of the *R* gene because one does not know the fraction of the many λ genomes present in an induced cell which are active. One can, however, make a crude comparison to another "late" protein (proteins) that should be formed in large amounts, the head protein (proteins) of the mature phage. The combined molecular weight of all proteins in a mature λ phage can be calculated to be about 33×10^6 daltons either from (a) the fact that the ratio of protein to DNA in mature λ is close to 1 (14) and the molecular weight of λ DNA is about 33×10^6 (31) or (b) from the density of the phage protein and the dimensions of the phage head and tail (32). Calculating that the head represents about 80% of the total protein, one obtains 26×10^6 daltons for the protein in the head. The 6×10^4 lysozyme molecules per cell represent some 1×10^9 daltons, or the equivalent in mass to the protein in about 40 phage heads.

This number is to be compared with the 100 mature phage produced per induced cell under these conditions. The comparison is not strict because we are ignorant of the number of phage head subunits that were synthesized but do not appear in the mature phage and the number of λ -lysozyme molecules hidden from the assay procedure by inactivation or adsorption to cell walls. However, even this crude comparison suggests that such disparate "late" proteins as λ -lysozyme and head protein (proteins) are synthesized at rates that differ by less than an order of magnitude, a suggestion that is somewhat surprising in view of the massive amounts of T4 head protein synthesized in *E. coli* infected with that phage (33).

Finally, it should be noted that approximately 100 mg of λ -lysozyme are obtained from the purification sequence given in Table I. This can be accomplished in about 2 weeks by one person. Thus, the amounts necessary for an analysis of

primary structure and for studies of crystallization properties can be obtained in reasonable times.

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