JOURNAL OF VIROLOGY, Aug. 1967, p. 701-710
Vol. 1, No. 4

Characteristics of a Lytic Enzyme Induced by Bacteriophage Infection of *Micrococcus lysodeikticus*[1]

J. M. GOEPFERT[2] AND H. B. NAYLOR

Department of Microbiology, Division of Biological Sciences, Cornell University, Ithaca, New York

Received for publication 1 April 1967

A lytic enzyme induced in *Micrococcus lysodeikticus* strain 1 by infection with N1 bacteriophage was purified 45- to 50-fold by ammonium sulfate precipitation, acid precipitation, and selective adsorption of contaminating proteins with calcium phosphate gel. The optimal *p*H for activity of the enzyme was 6.5 to 7.0. Maximal activity occurred at 45 to 50 C and at an ionic strength of 0.06. The enzyme had a limited specificity and lysed cell walls of *M. lysodeikticus* with the release of dinitrofluorobenzene reactive groups. Living cells were lysed in the absence of phage; however, the rate of lysis increased when phage was present in excess of 10 particles per bacterial cell. Young cells were most sensitive, and the sensitivity decreased to a minimum with stationary-phase cells. Acting synergistically, lysozyme and the N1-induced lysin caused lysis of cells which were resistant to either enzyme acting independently. The N1 lysin did not exhibit proteolytic activity.

Sertic (23) was the first to describe a lytic enzyme associated with bacteriophage infection. The subject lay dormant until 1945, when Anderson (3) studied ultraviolet-irradiated T bacteriophage preparations. More recently, many phage-associated lytic systems have been described (2, 4, 8, 9, 13, 15, 16, 20). The possibility of using these enzymes to study the chemical structure of the bacterial cell wall has greatly increased the interest in these enzymes.

Phage-associated lysins may be conveniently divided into two general classes. The first class consists of enzymes which depolymerize the capsular material surrounding some bacteria. The second group is comprised of the majority of the lysins studied thus far. Their action is exerted on the bacterial cell-wall.

Phage-induced lysins have been isolated from the medium after lysis of infected cells, and from the bacteriophage particles themselves. Murphy (15, 16) compared the enzyme found in lysates with the one isolated from phage particles and found them to differ in several properties. He suggested that the latter aided in the infective process, whereas the former was responsible for lysis of cells at the end of the latent period. The question of how the lytic enzyme is able to cross the cell membrane to act on its substrate remains unanswered.

On the basis of their site of action on the cell wall, phage lysins may again be divided into two types. The "backbone" splitting enzymes which act on the amino sugar linkages form one group, and the enzymes which cleave amide or peptide bonds, or both, fall into the second group.

This report describes the characteristics of a cell wall-digesting enzyme obtained from the lysates of phage-infected cultures of *Micrococcus lysodeikticus*.

MATERIALS AND METHODS

Bacterial cultures. M. lysodeikticus strain 1 (ML-1) as described by Naylor and Burgi (17) was the organism used for phage and enzyme production. It is highly sensitive to both lysozyme and N1 bacteriophage. *M. lysodeikticus* strain BLR was originally isolated by Brumfitt et al. (5), and was obtained from J. T. Park. It is partially resistant to lysozyme and is insensitive to N1 bacteriophage.

Bacteriophage. Bacteriophage N1 was originally isolated from sewage. Plated on ML-1, the plaques formed have a clear central zone 3 to 4 mm in diameter surrounded by a halo which increases in width with time of incubation. Bacteriophage titrations were performed by the overlay method of Adams (1).

[1] Submitted by the senior author in partial fulfillment of the requirements for the Ph.D. degree.

[2] Present address: Food Research Institute, University of Wisconsin, Madison 53706.

Media. The broth used for daily transfer of bacterial cultures consisted of 1.0% peptone, 0.5% yeast extract, 0.1% glucose, and 0.5% NaCl. The *p*H was 7.5. This was dispensed at the rate of 25 ml per 125-ml Erlenmeyer flask prior to autoclaving. When the BLR strain was grown in the presence of lysozyme, suitable dilutions of a 1% solution of the enzyme sterilized by filtration were added to the autoclaved broth to yield final concentrations of 1, 2, 5, or 10 μg/ml. Transfers were made daily and cultures were incubated at 30 C on a reciprocal shaker. Stock cultures were maintained on slants having the same composition as the transfer broth with the addition of 1.5% agar. Stock cultures were stored at 5 C and transferred at 6-month intervals. The broth used for production of high-titer bacteriophage lysates and lytic enzyme consisted of 1.5% peptone, 0.7% yeast extract, 0.2% glucose, and 0.2% NaCl in distilled water. This is referred to as enzyme production broth (EPB).

Preparation of phage lysates. A 5-ml amount of a 24-hr culture of ML-1 was inoculated into 100 ml of EPB in a 2-liter flask. This was incubated for 16 to 24 hr at 30 C on a reciprocal shaker. At this time, the culture was added to 400 ml of EPB in a baffled 2-liter flask. The appropriate volume of N1 phage suspension was added, and the flasks were incubated on a rotary shaker at 30 C until lysis was complete (usually 3 to 4 hr). The crude lysates were stored at 5 C until used.

Assay for lytic enzyme. A 0.9-ml sample of enzyme preparation was mixed with 0.1 ml of Difco Lysozyme Substrate suspension in a silica absorption cell to give an initial optical density of 0.8. The decrease in optical density was measured at 650 mμ in a Beckman model DU spectrophotometer equipped with an automatic recorder. The optical density was recorded every 10 sec. A graph of optical density versus time was obtained which was examined for the 2-min interval during which the greatest decline in optical density occurred. The slope of the curve during this period was determined, and a unit of enzyme activity was defined as that quantity of enzyme which would effect a decrease of 0.01 optical density units per minute. All assays were made at 37 C and *p*H 6.5 unless otherwise indicated.

Preparation of purified phage concentrates. Crude lysates were prepared in the manner described above. The lysates were treated with a few crystals of deoxyribonuclease to destroy the viscosity. The resulting lysate was then mixed with Volclay bentonite (SPV 90) obtained from the American Colloid Co., Skokie, Ill., at the rate of 2 g per 100 ml and was stirred for 30 min. The bentonite adsorbed essentially all of the lytic enzyme and much of the debris resulting from lysis of the cells, but none of the bacteriophage. The mixture was centrifuged at 5,800 × *g* for 15 min and the supernatant fluid was decanted. This was then centrifuged at 44,000 × *g* in a Spinco model L ultracentrifuge for 1 hr, and the supernatant fluid was discarded. The sediment from 160 ml was resuspended in 20 ml of diluent containing 0.1% peptone and 0.001 M $MgSO_4$, and then was filtered through a Selas filter (02 porosity) that was pretreated with 0.5 M $NaHCO_3$ to prevent adsorption of the phage on the porcelain.

Preparation of cell walls. All cell walls used were prepared by the method of Cummins and Harris (7).

Formylation of cell walls. Cell walls were formylated according to the method of Perkins (18).

Acetylation of cell walls. The method used was that of Heymann et al. (12). *O*-acetyl groups were removed by incubating the treated cell walls in 0.1 M glycine-NaOH buffer at *p*H 10.6.

Dinitrophenylation of cell walls. A 10-mg sample of cell walls was suspended in 2 ml of 1% (w/v) $Na_2B_4O_7 \cdot 10H_2O$ solution. To this was added 0.25 ml of dinitrofluorobenzene (0.1 M in absolute ethyl alcohol), and the mixture was heated at 60 C for 30 min. The walls were removed by centrifugation and washed successively with absolute ethyl alcohol-water (2:1, v/v), absolute ethyl alcohol, and water. The dinitrophenylated walls were suspended in distilled water and stored in the dark at 5 C until needed.

Determination of free NH_2 groups and amino sugars. The methods used were those of Ghuysen and Strominger (11).

Assay for proteolytic activity. A 0.5% solution of casein or serum albumin was autoclaved at 121 C for 20 min; 8 ml of sterile substrate was added to 2 ml of partially purified enzyme preparation, and the mixture was incubated at 37 C. Portions (1 ml) were withdrawn periodically, added to 9 ml of 10% trichloroacetic acid, and allowed to stand at room temperature for 1 hr. The resulting precipitate was removed by centrifugation and filtration, and the supernatant liquid was adjusted to *p*H 10.5 with 10 N KOH. Trichloroacetic acid-soluble protein was then determined by the method of Lowry et al. (14).

RESULTS AND DISCUSSION

Purification and properties of the lytic enzyme. Earlier, we reported that the N1 induced lytic enzyme could be partially purified by use of ammonium sulfate precipitation and subsequent selective adsorption to and elution from calcium phosphate gel (Goepfert and Naylor, Bacteriol. Proc., p. 142, 1964). This procedure resulted in a 37-fold purification, but the reliability varied with each lot of calcium phosphate gel. For this reason, the following procedure, which yielded more reproducible results, was established. The result of a typical purification is summarized in Table 1.

To a crude lysate containing 15.5 units of lytic activity per ml, several small crystals of deoxyribonuclease were added, and the mixture was incubated at 37 C for 20 min. At that time, the nonviscous lysate was chilled to 0 C, and solid $(NH_4)_2SO_4$ was added until 20% saturation was achieved. The fine precipitate which resulted was removed by centrifugation, and the supernatant fluid was adjusted to 40% saturation with $(NH_4)_2SO_4$ (no corrections were made for changes in volume). The precipitate was collected by

TABLE 1. *Purification of lytic enzyme*

Fraction	Activity	Protein	Specific activity	Relative purification	Yield
	units/ml	*mg/ml*			%
Crude lysate	15.5	9.8	1.58	—	100
20–40% $(NH_4)_2SO_4$	17	0.95	17.8	11.3	54.8
pH 5.2 supernatant fraction	12.5	0.55	24.7	15.6	40.3
*p*H 4.8–5.2 fraction	10	0.20	50	31.6	32.2
$CaPO_4$ gel supernatant fraction	9.5	0.128	74.2	47	30.6

centrifugation and resuspended in 0.5 volume of 0.01 M phosphate buffer at *p*H 6.5. The *p*H was adjusted to 5.2 with 2 N HCl, and the resulting precipitate was removed and discarded. The *p*H of the supernatant fraction was lowered to 4.8 with 2 N HCl; the precipitate was collected by centrifugation and suspended in 0.1 ionic strength phosphate buffer at *p*H 5.7. Calcium phosphate gel (Sigma Chemical Co., St. Louis, Mo.) was added at a ratio of 2.5 mg of gel per mg of protein, and the mixture was stirred for 30 min. Centrifugation removed the gel, leaving the lytic agent in the supernatant fluid. The anomalies arising from differences in adsorption by different lots of calcium phosphate gel were circumvented by this procedure. With this method, a 47-fold increase in specific activity with an overall yield of 30.6% was attained. In addition, the N1 bacteriophage titer was lowered from an initial 5×10^{10}/ml to approximately 1×10^6/ml.

Figure 1 shows the effect of *p*H on the activity of the lysin. The enzyme was active over a wide range of *p*H values and had an optimum between *p*H 6.5 and 7.0.

The lytic activity increased with the temperature (Fig. 2) to a maximum between 45 and 50 C. The heat stability of the enzyme at various *p*H values was determined (Fig. 3) by heating samples of the partially purified preparation to 45 C for 5 min and then quickly cooling them to 0 C prior to readjusting to *p*H 6.5 for assay of residual activity. It had been previously determined that no change in *p*H occurred during heating, although some of the *p*H values were outside the effective range of the phosphate buffer system in which the enzyme was suspended. Further studies have shown that the enzyme is most stable to heat at *p*H 8. All lytic activity was destroyed by heating the enzyme to 55 C for 5 min, regardless of the *p*H of the menstruum.

The enzyme could not be sedimented by centrifugation at 100,000 × *g* for 24 hr. Column chromatography of the partially purified preparation on Bio-Gel polyacrylamide indicated that the lytic enzyme is a relatively small protein (molecular weight, 30,000 to 60,000).

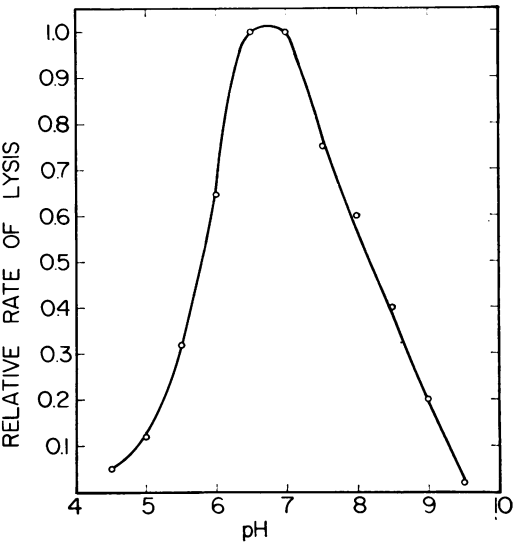

FIG. 1. *Effect of pH on rate of lysis of ML-1 cells by N1-induced enzyme (30 C).*

Our previous work disclosed that the N1 induced lysin has a nonspecific requirement for divalent cations. At that time no data were obtained to indicate that monovalent ions were either stimulatory or inhibitory. In the present study, purified enzyme preparation was exhaustively dialyzed at 1 C against 0.0001 M phosphate buffer at *p*H 6.5. Different levels of NaCl were then added back to determine the optimal ionic strength for enzyme activity. As depicted in Fig. 4, lytic activity increased with the ionic strength to a value of 0.06 and thereafter decreased slightly.

The effects of various chemical and physical treatments on the lysin are presented in Table 2. The enzyme was sensitive to each of the proteases tested. Chelating agents for divalent cations inhibited lysis but the inhibition was reversed by Ca^{++}, Mg^{++}, or Mn^{++}. Inactivation by reagents that react with sulfhydryl groups was reversed by cysteine and mercaptoethanol. The enzyme was not sensitive to ultraviolet light and lytic activity

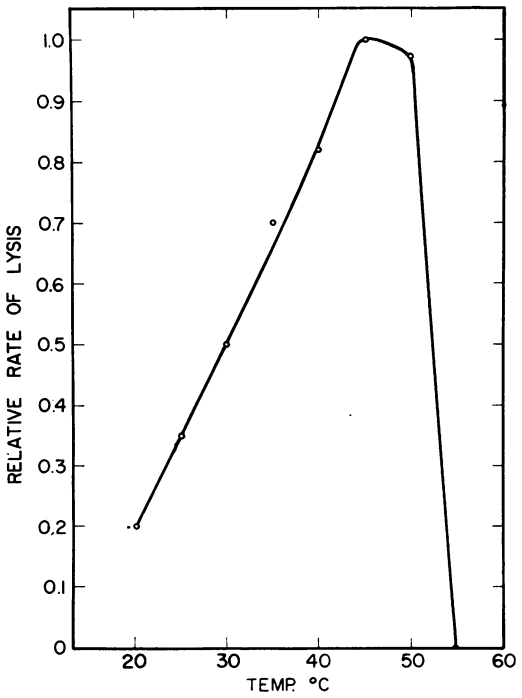

FIG. 2. *Effect of temperature on rate of lysis of ML-1 cells by N1-induced enzyme (pH 6.5).*

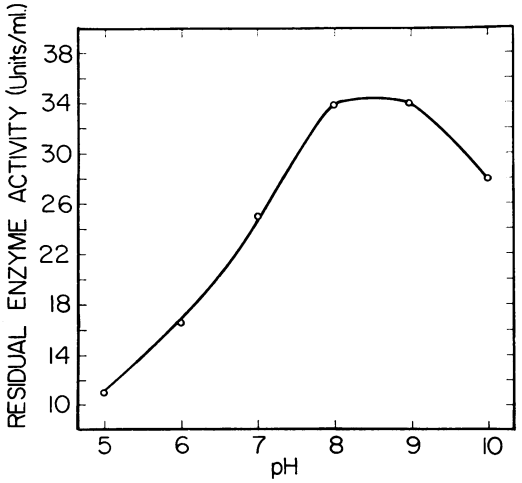

FIG. 3. *Effect of pH on the heat stability of N1-induced lytic enzyme. Samples of lytic enzyme were heated to 45 C for 5 min, and then quickly cooled to 0 C prior to adjusting to pH 6.5 for assay of residual activity.*

was not impaired by the addition of antiserum prepared against the intact phage particles.

Sensitivity of whole cells. The specificity of the enzyme is shown in Table 3. Gram-negative or-

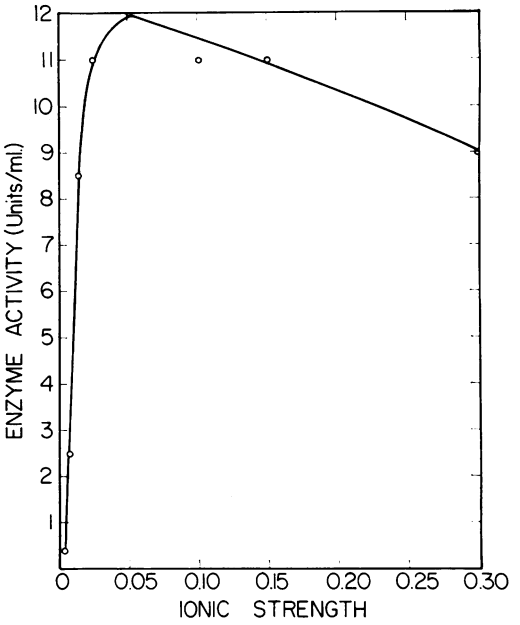

FIG. 4. *Effect of ionic strength on activity of N1-induced lytic enzyme. Ionic strength of dialyzed enzyme preparation was adjusted by the addition of NaCl.*

ganisms were not lysed by the N1-induced enzyme. Lysozyme-sensitive organisms, such as *Bacillus megaterium* and *Sarcina lutea*, were insensitive to the phage-induced lysin. The two strains of *Sarcina* found to be sensitive to the enzyme could also be infected by N1 bacteriophage and appeared to be very closely related to *M. lysodeikticus*. Possible explanations for this limited specificity are discussed below.

Sensitivity of ML-1 to lysis by the phage-induced enzyme varied with the age of the culture (Fig. 5). Young, actively growing cells were most sensitive to lysis. This sensitivity decreased with the age of the culture and reached a minimum when the cells entered the stationary phase of growth (about 18 hr under the conditions employed).

To test the possibility that N1 phage is required for lysis of the living cells, suitable dilution of a purified phage suspension containing 4×10^{12} infectious particles/ml were added to a 24-hr culture of ML-1 at the rate of 0.1 ml of phage suspension to 0.1 ml of culture. These mixtures were incubated at 37 C for 5 min, and then 0.8 ml of N1 enzyme preparation (6 units/ml) was added to each. The results (Fig. 6) show that the enzyme preparation caused rapid lysis to occur and that no lysis could be detected in the absence of enzyme even when phage to cell ratios as high as 1,000:1 were employed. Enzymatic lysis was

TABLE 2. *Action of various agents on N1 lysin*

Agent	Concn	Effect
Ethylenediaminetetraacetate	$1.6\text{–}3.3 \times 10^{-3}$ M	Complete inhibition[a]
Na citrate	0.01 M	Complete inhibition[a]
Iodoacetate	0.01 M	Complete inhibition[a]
p-Chloromercuribenzoate	3×10^{-5} M	Complete inhibition[a]
H_2O_2	0.02 M	Complete inhibition[a]
Pepsin	0.05 mg/ml	Complete inhibition[a]
Trypsin	0.05 mg/ml	Complete inhibition[a]
Papain	0.05 mg/ml	Complete inhibition[a]
Deoxyribonuclease	1 mg/ml	No effect
Ribonuclease	1 mg/ml	No effect
N1 antiserum		No effect
UV light	20 min at 30 cm	No effect

[a] Complete inhibition is defined as loss of ability of the N1 lysin to cause any reduction in turbidity of ML-1 within 15 min under the test conditions described in Materials and Methods.

stimulated, however, with a phage-to-cell ratio of 10:1, and even greater stimulation was observed when the ratio was increased to 100:1. Added N1 phage failed to stimulate lysis of heat-killed ML-1 cells, although these cells were more sensitive than unheated cells to lysis by the enzyme preparation.

The action of the N1-induced lysin was compared with egg white lysozyme on the partially lysozyme-resistant BLR strain of *M. lysodeikticus*. The results (Fig. 7) show that neither N1 lysin nor lysozyme (0.5 μg/ml) had much effect on BLR. However, when the two enzymes were added together prior to adding the BLR cells, a synergistic action resulted which caused rapid lysis to occur. The synergistic phenomenon was also observed when ML-1 was the test organism. To determine whether or not the N1 enzyme preparation contained a protein other than the lysin, capable of facilitating lysozyme action, samples of the preparation were singly subjected to several treatments (trypsin, 0.5 mg/ml; ethylenediaminetetraacetate, 5×10^{-3} M; and heating to 55 C for 5 min) known to inhibit or destroy the phage-induced lysin. The treated preparations were then tested for their effect on lysis of BLR by lysozyme. No stimulation was observed, although it must be pointed out that the existence of a protein other than the N1 lysin which facilitates lysozyme action cannot be unequivocally ruled out.

Whereas living BLR cells were relativly insensitive to lysis by either phage lysin or lysozyme, the situation changed when the cells were heat-killed (Fig. 8). Boiling the cells for 5 min caused them to become extremely sensitive to lysis by the N1 enzyme and concomitantly more resistant to lysis by lysozyme. This property permitted the development of a method for detecting intracellular enzyme production during the latent period of infection with N1.

TABLE 3. *Sensitivity of various organisms to N1 lytic enzyme*

Organism tested	Sensitivity to N1 lytic enzyme[a]
Aerobacter aerogenes	−
Proteus vulgaris	−
Escherichia coli	−
Pseudomonas aeruginosa	−
Serratia marcescens	−
Streptococcus lactis	−
S. faecalis	−
Lactobacillus arabinosus	−
Bacillus cereus	−
B. subtilis	−
B. polymyxa	−
B. megaterium	−
Staphylococcus aureus	−
Sarcina lutea	−
Micrococcus lysodeikticus	+
Sarcina flava	+
S. subflava	+

[a] An organism is regarded as insensitive when 20 units of N1 lysin fails to cause a 5% decrease in turbidity when assayed under the conditions described in Materials and Methods.

A 20% inoculum of ML-1 in EPB was infected with sufficient N1 phage suspension to supply six infectious particles per cell. The infected culture was incubated at 30 C on a reciprocal shaker. Samples were withdrawn periodically, and the cells were lysed with lysozyme (10 μg/ml of cell suspension). These samples were then assayed for phage lysin with heat-killed BLR cells as the substrate. Suitable dilutions of the samples were also assayed for infectious phage particles on plates seeded with ML-1. Assays for extracellular

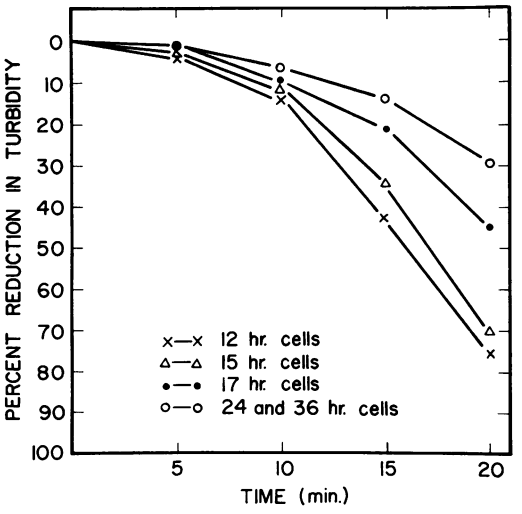

FIG. 5. *Effect of age of cells on sensitivity to N1-induced lytic enzyme. Assayed at pH 6.5 and 37 C. Initial turbidity of each culture was adjusted to 0.7 in the test system.*

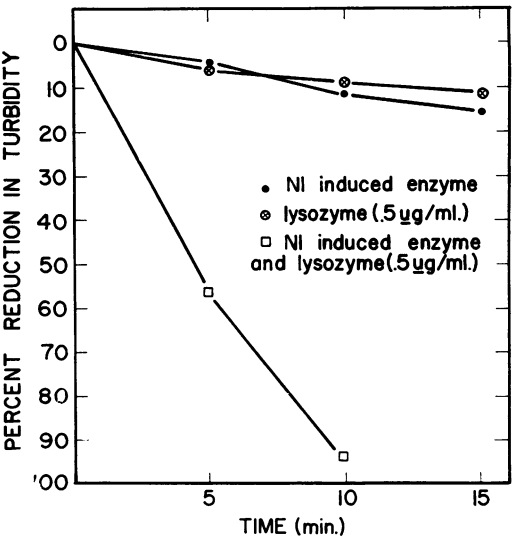

FIG. 7. *Synergistic effect of lysozyme and N1-induced lytic enzyme on BLR.*

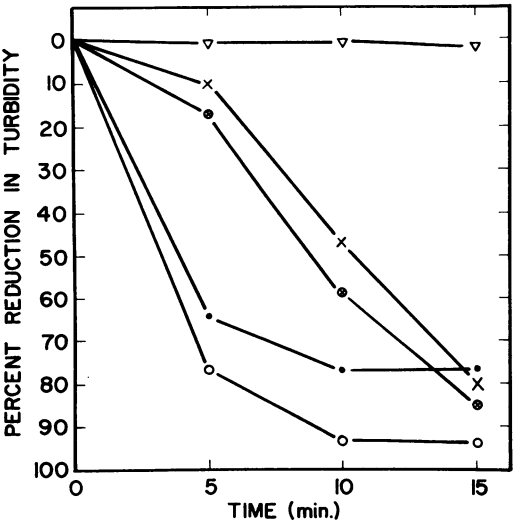

FIG. 6. *Stimulatory effect of N1 phage on lysis of ML-1 by N1-induced lytic enzyme. Symbols: ▽, N1 phage (phage-to-cell ratio, 1,000:1); ○, N1 enzyme + phage (phage-to-cell ratio, 100:1); ⊗, N1 enzyme + phage (phage-to-cell ratio, 10:1); ×, N1 enzyme (phage-to-cell ratio, 1:200); ●, N1 enzyme, with or without added phage, heat-killed cells.*

lytic activity were made on the supernatant fluids after centrifuging (identical samples which received no lysozyme treatment) to sediment the intact cells. The results (Fig. 9) show that, under the experimental conditions used, enzyme production could be detected in infected cells approximately 30 min after infection. This just preceded the formation of the first infectious phage particles. The enzyme could not be detected extracellularly until approximately 105 min after infection.

Cell wall studies. To determine the nature of the substrate in the cell wall being attacked by the N1 lysin, the following experiment was performed. A 4-mg amount of ML-1 cell walls (prepared as described above) was suspended in 2 ml of purified N1 enzyme preparation. Samples were removed periodically and tested for amino sugar and free NH_2 group release. The lysin did not release detectable amounts of amino sugar, but it did release free NH_2 groups at a linear rate for the first hour and more slowly thereafter (Fig. 10). Salton (22) found that 400 to 500 mμmoles of free NH_2 groups are present per mg of *M. lysodeikticus* cell walls. As shown by Perkins (18), these free NH_2 groups can be selectively blocked with formyl groups. When the free NH_2 groups of ML-1 cell walls were formylated prior to exposure to the N1-induced lysin, the amount of NH_2 groups liberated was reduced to about 450 mμmoles per mg of cell walls. Similar treatment of walls prior to digestion by lysozyme reduced the NH_2 group release from 450 to 45 mμmoles per mg of cell walls. The N1 enzyme released NH_2 groups to the same extent from lysozyme-treated walls as from the native walls.

Cell walls were formylated, acetylated, or dinitrophenylated to block free NH_2 groups before

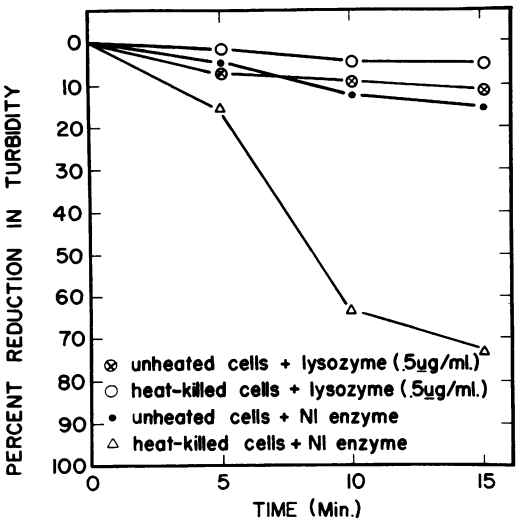

FIG. 8. *Effect of heat on sensitivity of BLR to lysis by lysozyme and N1-induced lytic enzyme.*

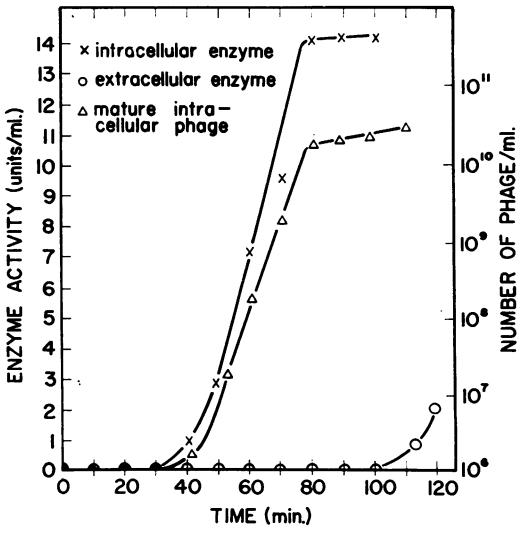

FIG. 9. *Phage and lytic enzyme production by ML-1 infected with N1 phage.*

exposure to the N1 enzyme. Figure 11 shows the effect of these treatments on the rate of cell wall lysis by the the N1 enzyme. Formylation and acetylation resulted in a stimulation of the rate of lysis when compared with untreated walls. Dinitrophenylation of the walls caused the walls to become almost totally resistant to lysis. This effect was probably due to steric hindrance brought about by the introduction of the relatively large dinitrophenol molecule.

Assays for proteolytic activity of the N1 enzyme preparation showed no release of trichloroacetic acid-soluble nitrogen from casein or serum albumin over a 12-hr period.

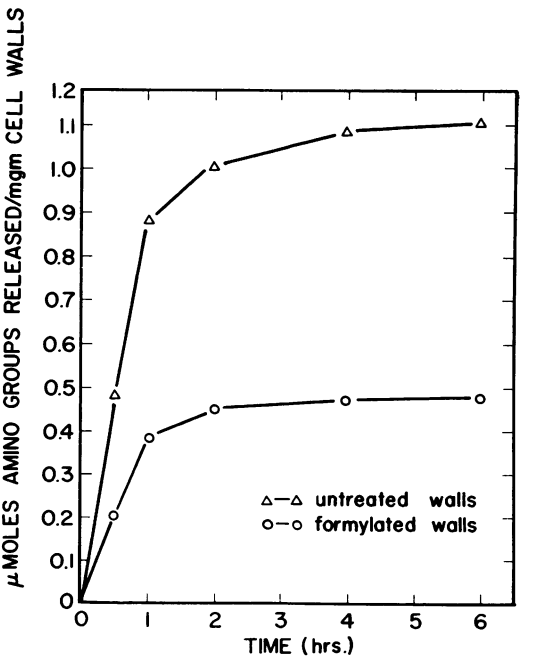

FIG. 10. *Effect of formylation on release of amino groups from ML-1 cell walls by N1-induced lytic enzyme.*

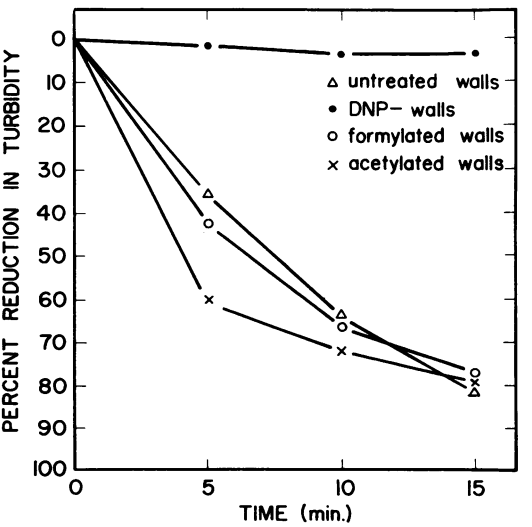

FIG. 11. *Effect of blocking free amino groups in ML-1 cell walls on rate of lysis by N1-induced lytic enzyme.*

DISCUSSION

According to the classical definition, the N1-induced lytic enzyme cannot be classified as a

"lysozyme," i.e. an enzyme which lyses cell walls with the release of reducing sugars and acetyl hexosamines. The N1 lysin does not liberate either Morgan-Elson or Elson-Morgan positive substances from cell walls. On the other hand, free NH_2 groups are released over and above the normal cell wall content of ϵ-NH_2 groups of lysine. This was determined both by the quantity of dinitrofluorobenzene (DNFB) reactive groups solubilized from "native" cell walls and by the release of NH_2 groups from cell walls in which the free NH_2 groups were blocked by treatment with hot formamide. The efficiency of this formylating procedure was checked by the determination of DNFB reactive groups solubilized by lysozyme digestion. Results showed that this treatment blocked over 90% of the available NH_2 groups in the cell wall.

Very recently, several other phage lysins have been shown to act on the peptide moiety of the peptidoglycan. N. E. Welker and L. L. Campbell (Bacteriol. Proc., p. 126, 1966) described a lytic enzyme produced by mitomycin C-induced cultures of a lysogenic strain of *B. stearothermophilus*. Cell wall lysis was accompanied by the release of DNFB reactive material. Indications were that the enzyme was an amidase cleaving the bond between the N-terminal amino acid of the peptide and the lactyl portion of muramic acid. The enzyme, virolysin, first reported by Ralston et al. in 1955 (20) was recently demonstrated by the same author (Bacteriol. Proc., p. 69, 1966) to lyse cell walls of *Staphylococcus aureus* by releasing small peptide fragments from the peptidoglycan. This action resulted in the destruction of phage receptor sites. C. C. Doughty, N. L. Shoemaker, and J. A. Mann (Bacteriol. Proc., p. 84, 1966) described a peptidase isolated from lysates of phage-infected *S. aureus* which liberated glycine, alanine, and glutamic acid from cell walls. These enzymes (and the N1 lysin) were isolated from phage-induced lysates and not from phage particles. The present authors were not able to demonstrate the presence of lytic enzyme in concentrated (10^{13} plaque-forming units/ml) suspensions of N1 phage. Multiple cycles of freezing and thawing, sonic treatment, and treatment of these concentrates with 8 M urea (37 C for 1 hr) failed to liberate detectable quantities of lytic enzyme.

It seems likely that the strict specificity of the N1-induced lysin is directly related to the amino acid composition of the peptidoglycan. In all likelihood, the sequence of amino acids is the final determinant. This would explain the resistance of microorganisms having cell wall peptide fractions only slightly different from *M. lysodeikticus*.

The lytic enzyme is a "late" enzyme of phage infection, a property which is characteristic of phage-induced lysins. Champe (6) reported that T2 lysozyme first appears in infected *Escherichia coli* at about the 8th min of a 25-min latent period. Virolysin is detectable (21) in a 40-min latent period between the 10th and 15th min. At 20 to 30 minutes after infection (about 50% of the latent period), the "soluble" lysin makes its appearance in *B. megaterium* (15).

It is of interest to contrast the N1 lysin with virolysin and the "soluble" enzyme. The latter two enzymes lyse living cells only if the homologous phage is present. The N1 lysin does not require the presence of phage to lyse living cells, although the rate of lysis is indeed stimulated when N1 phage is present at phage-to-cell ratios of 10:1 or greater. Three possible explanations may be considered. First, the phage may add to the total quantity of lytic enzyme present, either by carrying adsorbed enzyme or by having a lytic enzyme as an integral part of its structure. Second, phage infection of a cell may interfere with normal cell metabolism and exert an effect on enzymes involved with cell wall synthesis. Last, as proposed by Ralston and co-workers (19), the cell wall may contain a thermolabile inhibitor whose effect is overcome by phage adsorption. The first possibility may be ruled out for the N1-induced lysin on the basis that N1 phage alone could not effect lysis even when present at multiplicities greater than 1,000:1, and that there was no stimulation of enzymatic lysis of heat-killed cells by phage though the heat treatment did enhance susceptibility of the cells to the N1 lysin. There is no definitive evidence to rule out either of the latter two possibilities, though we tend to favor the second. There is a wealth of information proving that host cell metabolism is altered after phage infection. None of the work has been extended to the effect on cell wall synthesizing enzymes, but it would not be surprising to find altered rates of activity or synthesis of these enzymes. The increased sensitivity of heat-killed cells could be explained by this hypothesis, as these enzymes would most probably be destroyed by the heating process (boiling for up to 15 min in some cases).

Resistance to lysozyme (in *M. lysodeikticus*) can be attributed to an increased *O*-acetyl content of the cell wall (5). Although *O*-acetylation partially restricts lysis of cell walls by the N1 lysin, it appears that this is not the only factor (and probably not the main one) which is responsible for the resistance of BLR cells to the phage lysin. If it were, we would not expect boiling the cells for 5 min at *p*H 6.5 to restore sensitivity, as this treatment does not remove appreciable quantities

of hydroxyl-linked acetyl groups. If cleavage of these esters occurred, a simultaneous return to lysozyme sensitivity should result; this was not observed. Heat-killing the cells does not return them to the level of extreme sensitivity characteristic of the lysozyme-sensitive strain from which the BLR organism was derived. In addition, partial removal of *O*-acetyl groups by treatment of the cells for 1 hr in buffer at *p*H 10.6 and 37 C results in only a very slight increase in sensitivity. Thus, it would appear that a thermolabile material or chemical linkage is responsible for resistance to lysis by the N1 lysin.

The synergistic action of the N1 lysin and lysozyme raises several questions. If this phenomenon had been observed only with ML-1, it would not seem unusual. Its appearance, however, when BLR cells are used for assay cannot be explained so readily. Extracts of uninfected BLR cells did not stimulate lysozyme-induced lysis of this strain. The possibility that phage infection resulted in the synthesis of a de-*O*-acetylase cannot be eliminated from consideration. Attempts to demonstrate that a stimulatory substance other than the N1 lysin was present in phage lysates were not successful by the methods employed in this study. Examination of the digestion products, of the BLR cell walls, after treatment with the N1 lysin-lysozyme mixture, for the ester-linked acetyl groups would have determined the presence or absence of a de-*O*-acetylase in the preparation. Unfortunately, this was not done. When the BLR cells were grown in the presence of lysozyme, they could be washed repeatedly with distilled water or phosphate buffer without altering sensitivity to N1 lysin. The susceptibility of these cells was found to be a function of the lysozyme concentration in the medium in which they were grown. These findings suggest that the role of the lysozyme may be to cover the chemical group in the cell wall which is responsible for resistance of these walls to the N1 lysin.

The lack of proteolytic activity by the N1 lysin indicates that it is not one of the class of cell wall lytic enzymes recently reported by Ensign and Wolfe (10).

Lysis of ML-1 is due to the peptidase activity of N1 lysin. The exact site of cleavage has not been determined, but preliminary results suggest that the enzyme is not an amidase.

ACKNOWLEDGMENT

This investigation was supported by Public Health Service training grant GM 1385-06 from the National Institute of General Medical Sciences.

LITERATURE CITED

1. ADAMS, M. H. 1959. Bacteriophages. Interscience Publishers, Inc. New York.
2. ADAMS, M. H., AND B. H. PARK. 1956. An enzyme produced by a phage host-cell system. II. The properties of the polysaccharide depolymerase. Virology **2**:719–736.
3. ANDERSON, T. F. 1945. On a bacteriolytic substance associated with a purified bacterial virus. J. Cellular Comp. Physiol. **25**:1–15.
4. BRUMFITT, W. 1960. Studies on the mechanism of adsorption and penetration by bacteriophage. J. Pathol. Bacteriol. **79**:1–9.
5. BRUMFITT, W., A. C. WARLAW, AND J. T. PARK. 1958. Development of lysozyme resistance in *Micrococcus lysodeikticus* and its association with an increased O-acetyl content of the cell wall. Nature **181**:1783–1784.
6. CHAMPE, S. P. 1963. Bacteriophage reproduction. Ann. Rev. Microbiol. **17**:87–114.
7. CUMMINS, C. S., AND H. HARRIS. 1956. The chemical composition of the cell wall in some gram-positive bacteria and its possible value as a taxonomic character. J. Gen. Microbiol. **14**: 583–600.
8. DOUGHTY, C. C., AND J. A. HAYASHI. 1962. Enzymatic properties of a phage-induced lysin affecting group A streptococci. J. Bacteriol. **83**: 1058–1068.
9. EKLUND, C., AND O. WYSS. 1962. Enzyme associated with bacteriophage infection. J. Bacteriol. **84**:1209–1215.
10. ENSIGN, J. C., AND R. S. WOLFE. 1966. Characterization of a small proteolytic enzyme which lyses bacterial cell walls. J. Bacteriol. **91**:524–534.
11. GHUYSEN, J. M., AND J. L. STROMINGER. 1963. Structure of the cell wall of *Staphylococcus aureus* strain Copenhagen. I. Preparation of fragments by enzymatic hydrolysis. Biochemistry **2**:1110–1119.
12. HEYMANN, H., J. M. MANNIELLO, AND S. S. BARKULIS. 1964. Structure of streptococcal cell walls. III. Characterization of an alanine-containing glucosaminyl-muramic acid derivative liberated by lysozyme from streptococcal glycopeptide. J. Biol. Chem. **239**:2981–2985.
13. HUMPHRIES, J. C. 1948. Enzymic activity of bacteriophage-culture lysates. I. A capsule lysin active against Klebsiella pneumoniae type A. J. Bacteriol. **56**:683–693.
14. 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**:265–275.
15. MURPHY, J. S. 1957. A phage-associated enzyme of *Bacillus megaterium* which destroys the bacterial cell wall. Virology **4**:563–581.
16. MURPHY, J. S. 1961. An agent derived from *Bacillus megaterium* phage G which dissolves the bacterial cell wall. Virology **11**:510–513.
17. NAYLOR, H. B., AND E. BURGI. 1956. Observa-

tions on abortive infection of *Micrococcus lysodeikticus* with bacteriophage. Virology **2:** 577–593.

18. PERKINS, H. R. 1965. The action of hot formamide on bacterial cell walls. Biochem. J. **95:**876–882.
19. RALSTON, D. J. 1963. Staphylococcal sensitization: specific biological effects of phage K on the bacterial cell wall in lysis-from-without. J. Bacteriol. **85:**1185–1193.
20. RALSTON, D. J., B. BAER, M. LIEBERMAN, AND A. P. KRUEGER. 1955. Virolysin: A virus-induced lysin from staphylococcal phage lysates. Proc. Soc. Exptl. Biol. Med. **89:**502–507.
21. RALSTON, D. J., B. BAER, M. LIEBERMAN, AND A. P. KRUEGER. 1961. Virolysin, a virus induced lysin: Its appearance and function in phage infected staphylococci. J. Gen. Microbiol. **24:**313–325.
22. SALTON, M. R. J. 1964. The bacterial cell wall. Elsevier Publishing Co., Amsterdam.
23. SERTIC, V. 1929. Origine de la lysine d'une race du bacteriophage. Compt. Rend. Soc. Biol. **100:**477–479.