ENZYMATIC PROPERTIES OF A PHAGE-INDUCED LYSIN AFFECTING GROUP A STREPTOCOCCI

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ABSTRACT

DOUGHTY, C. C. (University of Illinois College of Medicine, Chicago) AND JAMES A. HAYASHI. Enzymatic properties of a phage-induced lysin affecting group A streptococci. J. Bacteriol. **83**:1058–1068. 1962.—Phage-induced lysis of group C streptococci releases into the medium a lysin which completely lyses group A streptococci. Partial purification of the lytic activity yields 47% of the original activity with a 17-fold purification. The activity was assayed by observing lysis of group A streptococci under standard conditions. The optimal pH range for lysis is from 6.0 to 6.7. A monovalent cation requirement satisfied by Na^+, K^+, or Li^+ is shown by the lysin. Lysis is stimulated by ethylenediaminetetraacetic acid (EDTA), chlortetracycline, streptomycin, and penicillin. It is inhibited by *p*-hydroxymercuribenzoate (*p*HMB), and the inhibition is reversed by cysteine. Other inhibitors include ristocetin A and specific antisera against the lysin. Isolated group A streptococcal cell walls are partially lysed by massive amounts of lysin. This partial lysis is not affected by EDTA, *p*HMB, chlortetracycline, streptomycin, or ristocetin A. It is concluded that the enzymatic process of lysis of isolated cell walls is not identical to the more complex process resulting in lysis of intact cells.

The lysis of group C streptococci after infection with their homologous bacteriophage results in the release of a lytic factor into the culture medium (Maxted, 1955; Krause, 1957). This lytic factor lyses group A streptococci effectively, and has been used to release various antigens from the cell walls of group A streptococci (Krause, 1957; Kantor and Cole, 1960; Krause and McCarty, 1961). It has also been used in hypertonic solutions to produce protoplasts and L forms of group A streptococci (Freimer, Krause, and McCarty, 1959). The latter investigators indicated the enzymatic character of the lytic factor and, indeed, achieved some degree of purification of the lytic factor using conventional methods of enzyme purification.

This paper describes some studies of the enzymatic properties of the lytic system. Purification and stability of the enzyme were investigated, as well as the conditions needed for optimal enzyme activity.

The study of inhibitors of the lytic process indicates that lysis of whole cells is inhibited by sulfhydryl reagents and by ristocetin A, although the lysis of isolated cell walls is not affected by those reagents. These facts indicate that lysis of cells is a multi-step process, where individual steps may be inhibited by different reagents.

MATERIALS AND METHODS

Bacterial cultures and bacteriophage. The organism used in the turbidimetric assay of lysin activity was a strain of type 14, group A, β-hemolytic *Streptococcus pyogenes*, designated S 23, which was obtained from Rebecca C. Lancefield, Rockefeller Institute for Medical Research. The group C streptococcal culture, designated 26RP66, and the C 1 bacteriophage, which was used in preparing lysin, were obtained through R. M. Krause, Rockefeller Institute for Medical Research.

Culture media. Difco Todd-Hewitt broth medium (30 g per liter) was employed for bacterial growth.

Bacteriophage plaque counts. An agar-base medium, which supported luxuriant growth of strain 26RP66, was obtained by addition of 1% yeast extract (Difco) and 2% agar to Todd-Hewitt broth. The bacteriophage dilutions were made in Todd-Hewitt broth and incubated in a water bath at 46 C. Melted agar was added to a concentration of 0.8%. Before mixing, an addition of 0.2 ml of the streptococcal culture

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was made. This semisolid agar was poured onto the surface of the Todd-Hewitt agar plates. After overnight incubation at 37 C, the plaques were counted.

Assay of lysin. Group A streptococci, strain S 23, were harvested after 16 hr of growth at 37 C in Todd-Hewitt broth. They were washed twice with distilled water and stored at −30 C in thick suspensions. Dilutions were made in 0.1 M phosphate buffer (pH 6.1) at the time of assay. Lysin solution (0.03 to 0.10 ml) was added to 10 ml of cells in 18-mm diameter tubes, and the turbidity decrease at 37 C was observed at 660 mμ in a Coleman Junior spectrophotometer. A second assay medium was 3% Todd-Hewitt broth made up in the above phosphate buffer.

A unit of lysin was defined as the decrease in optical density of 1.00 per min under standard assay conditions.

Lyophilization of the bacteriophage. Group C streptococci, strain 26RP66, in the logarithmic growth phase were infected with a tenfold excess of C 1 bacteriophage. After 5 min incubation at 37 C, to assure virus adsorption and penetration into the bacterial cell, the infected cultures were rapidly cooled to 0 C. After centrifugation at 10,000 × *g* for 10 min at 4 C, the sedimented bacterial cells were suspended in a minimal amount of Todd-Hewitt broth to which had been added an equal volume of sterile, heat-inactivated (56 C for 20 min) horse serum. The phage-infected bacteria were lyophilized in ampoules, sealed under vacuum, and stored at −30 C.

Preparation of specific antisera. Before immunization, the sera of the rabbits used gave negative precipitin tests against both the phage and the purified lysin. Antigen solutions of the phage and lysin were made to contain about 100 mg protein per ml of 0.85% saline. During the first week of immunization, the rabbits were injected intravenously on three successive days with 0.25 ml of antigen. During the second week, three intravenous injections of 0.5 ml were given. Three injections of 1.0 ml were given on successive days of the third and fourth weeks. During the fifth week, the rabbits were bled and immune sera obtained. Both antisera, against virus and lysin, gave positive precipitin reactions against antigen solutions containing 0.02 mg protein per ml.

RESULTS

Assay of lytic activity. The assay used was based upon the rate of lysis of group A streptococci by the lysin. Some of the variables affecting the lysis, and the assay, were studied early in these investigations.

Figure 1 shows how lysis depends upon pH. Optimal rates of lysis were observed to occur in

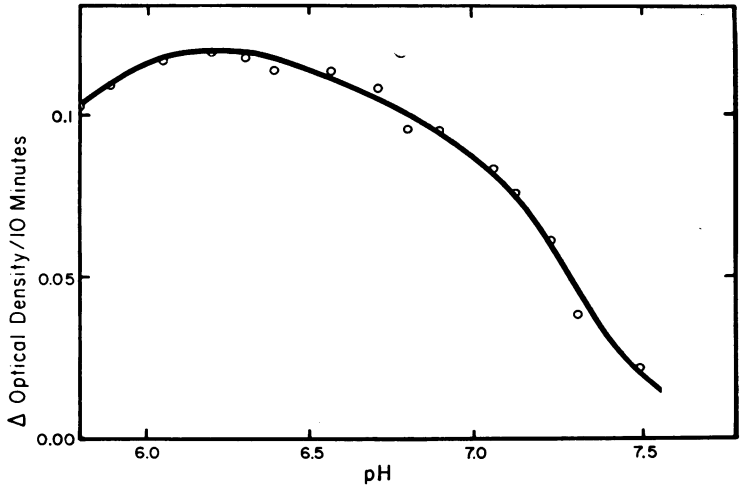

FIG. 1. *Effect of pH on lysis. Each sample contained group A streptococcal cells (initial OD, 0.150) in buffered Todd-Hewitt assay medium at the appropriate pH. The pH of each sample, measured before and after lysis, showed no change.*

0.10 M potassium phosphate buffer between pH 6.0 and 6.7.

The rate of lysis was shown to be proportional to the concentration of the lysin. Typical values are shown in Table 1 and illustrate the linear relationship which exists between lysin concentration and rate of lysis of group A streptococcal cells.

The specificity of the lysin was tested in a limited fashion. Freshly prepared lysin caused rapid lysis of all strains of group A streptococci tested. The group A strains tested included the following serological types: 1, 12, 14, 17, 23, 28, and 30. No significant differences were observed in rate of lysis among these strains. No difference in the rate of lysis was observed between a highly virulent group A strain, which had been serially passed through mice 43 times (Leedom and Barkulis, 1959), and its avirulent parent strain.

TABLE 1. *Effect of lysine concentration on the rate of bacterial lysis*

Lysin added*	Δ Optical density/min†
ml	
0.10	0.012
0.20	0.022
0.50	0.048
1.00	0.108

* Crude lysin used.

† Values obtained from the slope of the straight line portion of the curve.

When group C streptococcal strain 26RP66 was used as the test organism, lysis proceeded at a much slower rate (less than one-quarter) than lysis of group A streptococci. No measurable lysis was observed, even after 3 hr, in the two strains of group B (types 1a and 2), and in the two strains of group G (types 1 and 16) streptococci.

The physiological age of cells seems to be important in susceptibility to lysis. Cells which have been incubated in Todd-Hewitt broth at 37 C for several days do not lyse completely, and cells incubated over 5 days show almost no turbidity decrease, even when mixed with large amounts of lysin. Washed cells, harvested after overnight growth and stored at −20 C, retain their susceptibility to lysis for at least 3 months. However, upon prolonged standing at room temperature or 37 C, they show incomplete lysis when exposed to the lysin. It appears, then, that intact cells become less susceptible to the lysin as they become older and metabolically less active.

Preparation of lysin. 1) Effect of phage and host concentration on lysin titer:—Attempts were made to increase the yield of lysin produced by varying the virus and bacterial host-cell concentrations. In the first set of experiments, the host-cell concentration was increased to as much as five times the original concentration of bacterial cells in growing cultures of strain 26RP66. This was done by harvesting the group C 26RP66 streptococcal cells during the loga-

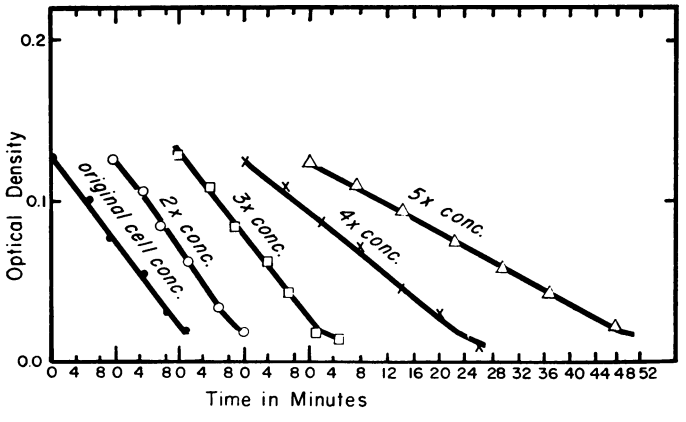

FIG. 2. *Cell concentration and lysin titer. Each sample contained 10 ml of a streptococcal suspension in Todd-Hewitt broth (0.130 OD) and 0.05 ml of crude lysin prepared from each of the indicated cell concentrations of 26 RP66 streptococci. A sliding time scale is employed in the figure.*

TABLE 2. *Purification of lysin*

Fraction	Activity (total units)	Yield	Protein	Specific activity*	Relative purification
		%	*mg*		
Crude lysate	117	100	1200	0.095	1.0
Protamine-treated supernatant	103	89	1090	0.095	1.0
$(NH_4)_2SO_4$ fractions					
0–25%	8.5	7	8	1.1	11.0
25–45%	46.7	40	29	1.6	17.0
45–60%	—	—	105	—	—
Above 60%	—	—	737	—	—

* Specific activity is expressed as optical density decrease per min for each mg of protein.

rithmic-growth phase and resuspending them in fresh Todd-Hewitt broth at 37 C. The same quantity of phage inoculum was added to each batch of cells. Lysis was completed in all of the tubes after 100 min, at which time all of the tubes were chilled to 0 C in an ice bath. A sample (0.05 ml) from each tube was then assayed for lysin titers. The results indicated that no increased titer was obtained by increasing the concentration of host cells up to three times their original concentration (Fig. 2). Equivalent lysin titers were obtained for each of the concentrations employed up to three times the original concentration of host cells. Above this concentration of host cells, a slightly decreased titer resulted; at four times the concentration, 10 units of lysin per ml resulted; at five times the concentration, 7 units per ml were obtained.

Similarly, in a second set of experiments, the virus concentration was increased and the host-cell concentration was maintained. The optimal conditions for lysin titer were obtained when about 10 virus particles per host cell were added. Further increases in the ratio of virus to host bacteria resulted in no significant increase in lysin titer.

2) Purification of lysin:—Preparation of a partially purified phage-induced lysin has been described by Krause (1958). In that preparation, the lytic activity was collected from a 0 to 50% saturated ammonium sulfate fraction precipitated directly from the crude phage lysate. Three days of storage at 4 C were required to precipitate the lytic activity. This investigation revealed that such long storage resulted in loss of as much as 90% of the total activity. This loss of activity could be minimized by first removing nucleic acid from the crude lysate by employing a protamine sulfate precipitation (Zelitch, 1955) followed by fractional precipitation of substances responsible for lytic activity with ammonium sulfate. Under these conditions, the time required to precipitate the cause of lytic activity with ammonium sulfate was reduced from 3 days to 10 min, and the precipitation was easily reproducible.

Crude phage lysate was prepared in Todd-Hewitt broth as described previously. Nucleic acid was precipitated from this fraction by addition of 10 ml of 1% protamine sulfate, in 0.1 M potassium phosphate buffer (pH 6.1), to each 100 ml of crude lysate. The protamine sulfate solution was slowly added to the lysate with constant stirring; the lysate was kept at 0 C in an ice bath. Immediately upon the addition of protamine sulfate, a stringy precipitate resulted. An increase in the 280 to 260 mμ absorbancy ratio was observed in the supernatant liquid after the protamine sulfate treatment, and 89% of the original lytic activity remained in the supernatant fluid.

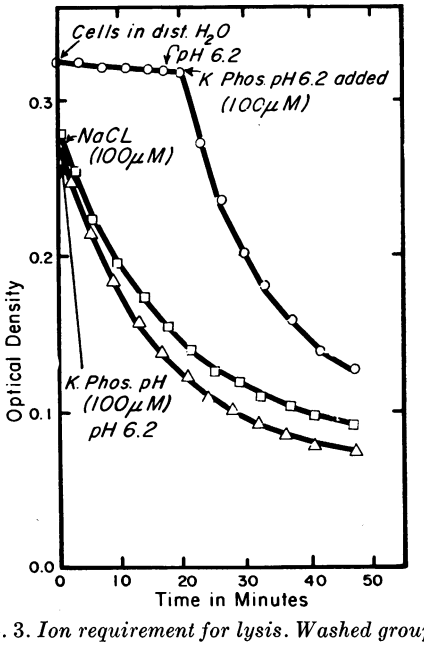

FIG. 3. *Ion requirement for lysis. Washed group A streptococcal cells suspended in distilled water, with addition of salt at the time indicated and 0.05 ml crude lysin added.*

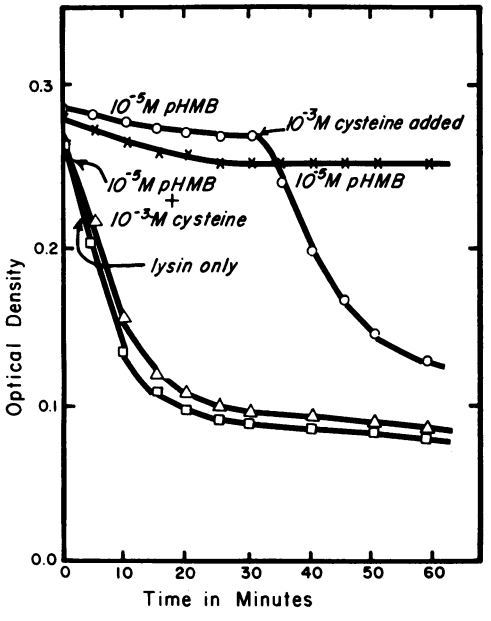

FIG. 4. *Lytic inhibition by p-hydroxymercuribenzoate and its reversal by cysteine. Standard assay conditions used with phosphate assay medium (0.05 ml crude lysin added).*

The ammonium sulfate fractions were obtained by slow addition of solid ammonium sulfate at 0 C. The precipitated fractions were redissolved in 0.1 M potassium phosphate (pH 6.1). As shown in Table 2, 47% of the original activity was collected in these fractions; 40% of the activity was present in the 25–45% $(NH_4)_2SO_4$ fraction. A 17-fold purification resulted in the latter fraction.

In some of the later experiments, increased stabilization during purification was obtained by addition of 0.1 M ethylendiaminetetraacetic acid (EDTA) to the crude lysate. The action of EDTA probably protects the lysin by chelation of toxic trace metals.

Inhibitors and activators of lysin. The effects of certain inhibitors and activators on the rate of lysis of group A streptococcal cells were studied using crude preparations of lysin, and further confirmed with purified lysin.

1) Effect of monovalent cations on lysis:—It was found that when lysin was added to twice-washed streptococcal cells suspended in distilled water, no lysis occurred. Although no buffer was added, the pH was 6.3, within the pH range for optimal lytic activity. Immediately upon addition of 100 μM of potassium phosphate (pH 6.1), lysis rapidly proceeded to completion (Fig. 3). A marked stimulation of rate of lysis by monovalent ions is indicated. Both sodium and potassium phosphate have equivalent effects in increasing the rate of lysis, and the same increased rates were observed when 100 μM of NaCl, KCl, or LiCl were added. When a similar amount of divalent cations ($MnCl_2$ or $CaCl_2$) was added, no significantly increased rate of lysis occurred.

It is concluded that monovalent cations (Na^+, K^+, or Li^+) are required for optimal rates of lysis. Divalent cations (Mn^{++} and Ca^{++}) have no significant effect on lysis, and neither do the anions (chloride or phosphate).

2) Effect of inhibitors on lysis:—Lysis of S 23 cells was almost completely inhibited by 10^{-5} M *p*-hydroxymercuribenzoate (*p*HMB). This inhibition could be reversed either at the time the lysin was added, by prior addition of cysteine, or after 30 min delay; in both cases the rate was the same as the rate of lysis without added *p*HMB (Fig. 4). Thus, the inhibition appeared to be completely reversed with no irreversible inactivation of lysin. When the cells were first incubated for 1 hr with 10^{-4} M *p*HMB, then

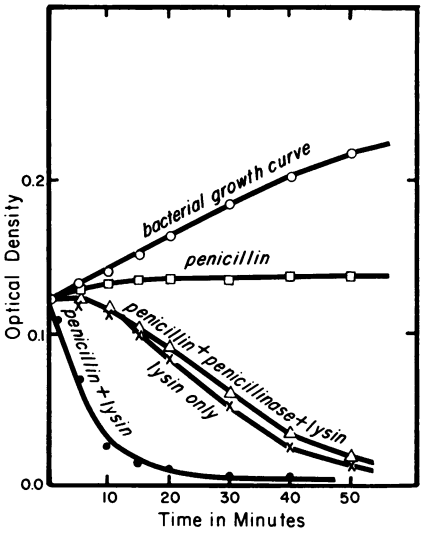

FIG. 5. *Effect of penicillin on lysis. Each tube contained S 23 cells suspended in 10 ml of buffered Todd-Hewitt assay medium. The following additions were made, where indicated on the figure: 100 units of penicillin, 0.05 ml of freshly prepared crude lysin, and 0.1 ml of penicillinase (Bacto-Penase concentrate).*

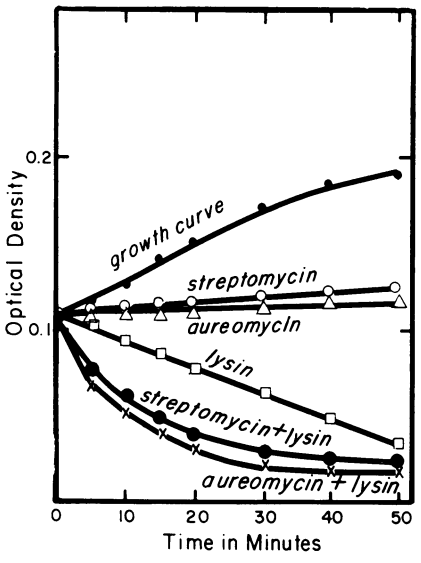

FIG. 6. *Effects of streptomycin and chlortetracycline on lysis. Each tube contained washed S 23 cells suspended in the buffered Todd-Hewitt assay medium. Where indicated, the following additions were made: 1.0 mg of streptomycin; 1.0 mg of chlortetracycline; and 0.05 ml of freshly prepared crude lysin.*

washed twice with water before addition of lysin, lysis proceeded at the normal rate. Apparently, no irreversible damage occurred on the surface of the cells to prevent lysis.

The rate of lysis was unaffected by either 10^{-3} M sodium arsenite, 10^{-3} M sodium arsenate, 2×10^{-2} sodium fluoride, 10^{-3} M iodoacetate, or 10^{-3} M atabrin. The rate was slightly increased by addition of 10^{-3} M *o*-phenanthrolene, possibly by chelation of trace metals. A slight increase in rate was observed after addition of 10^{-3} M sodium azide, 10^{-3} M potassium cyanide, or the strong reducing agent, 10^{-3} M sodium hydrosulfite.

3) Effect of antibiotics on lysin:—In studying the effects of antibiotics on rate of lysis, growing cells were suspended in Todd-Hewitt assay medium. It was then possible to establish, first, the effect of the antibiotic on growth of the bacteria, and second, the effect of the antibiotic on the lytic system.

Penicillin. The rate of lysis by the phage-induced lysin was increased two- to threefold by addition of penicillin G, 10 units per ml (Fig. 5). The control tube containing no lysin, and with penicillin added, showed bacterial growth inhibition, but no decrease in turbidity due to lysis. The control tube with no lysin and no penicillin added showed normal bacterial growth. When penicillinase (0.1 ml Bacto-Penase concentrate) was added prior to addition of lysin, no penicillin effect was observed. It has been suggested (Pollock, 1960) that the action of penicillinase on penicillin results in the cleavage of the β-lactam ring, thus resulting in the formation of penicilloic acid, which has no antibiotic properties. From the observations described above, penicillinase has no stimulatory effect on lysis of streptococci in Todd-Hewitt medium. The mechanism of the stimulation of lysis by penicillin is not fully explained.

Chlortetracycline and streptomycin. Upon addition of either chlortetracycline or streptomycin (0.1 mg per ml) and lysin to S 23 cells in potassium phosphate buffered Todd-Hewitt broth (pH 6.1), an increased rate of lysis was observed. This increased rate of lysis was three to four times greater than when lysin was added without antibiotic (Fig. 6).

The increase in lysis rate with chlortetracycline

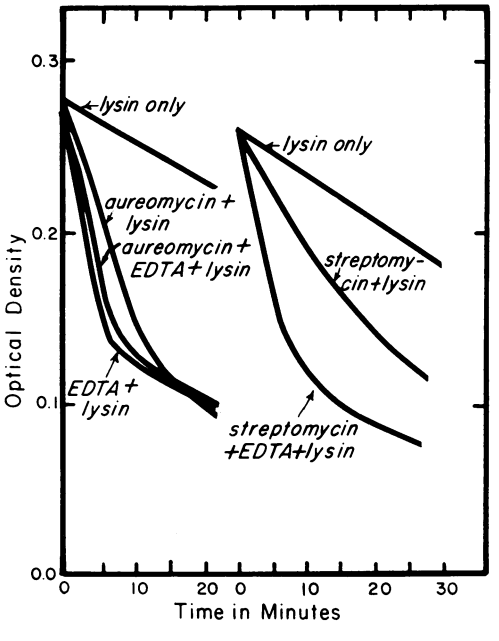

FIG. 7. *Comparison of effects of EDTA, chlortetracycline, and streptomycin on lysis. Standard assay conditions with buffered Todd-Hewitt broth. Additions the same as in Fig. 6, except EDTA at 10^{-3} M concentration. Crude lysin (0.05 ml) added.*

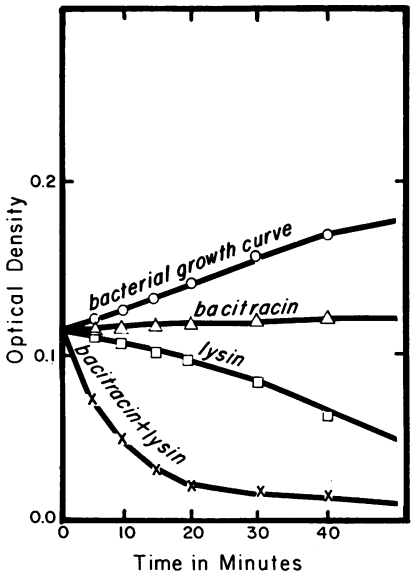

FIG. 8. *Effect of bacitracin on lysis. The buffered Todd-Hewitt assay medium was used. Where indicated, 0.03 ml of crude lysin and 1.0 mg of bacitracin were added*

was equivalent to the increased rate observed when 10^{-3} M EDTA was added, or when both EDTA and chlortetracycline were added to the lysis system (Fig. 7). The stimulation of lysis by addition of streptomycin was 50% of that resulting when either EDTA or EDTA and streptomycin were added.

Both chlortetracycline and streptomycin have been shown to be effective chelating agents for metallic ions (Albert and Rees, 1956; Foye et al., 1955). A possible explanation of their catalytic roles in lysis may depend on the chelation of an inhibitory metallic ion by the antibiotic.

Further experiments indicated the presence of an inhibitor in Todd-Hewitt broth which may be removed by chelation, as no stimulation of rate of lysis occurred in the absence of Todd-Hewitt broth when either antibiotic or EDTA was added to lysin, and washed cells suspended in phosphate buffer.

Bacitracin. The rate of lysis was increased four to five times by addition of 0.1 mg per ml of bacitracin (Fig. 8). It is possible that this effect may also depend upon formation of a metal-antibiotic complex, since bacitracin has been observed to precipitate with salts of heavy metals (Anker et al., 1948).

Ristocetin A. The antibiotic ristocetin A has been described by Phillip, Schenck, and Hargie (1956) as a carbohydrate-containing polypeptide. When this antibiotic was used in the presence of lysin, the results were at variance with the expected behavior. When ristocetin A was added to lytic systems in a concentration of 5×10^{-5} M, there was 90% inhibition of lysis (Fig. 9). When 1×10^{-4} M ristocetin was used without lysin, there was no significant difference observed between the sample and the normal control. When lysin and ristocetin were added together, at a ristocetin A concentration of $1\text{-}2 \times 10^{-4}$ M, there was a marked increase in turbidity as compared to the normal control.

4) Inhibition of lysis with specific antiserum:— Complete inhibition of lysis resulted when 0.5 ml of the high-titer antilysin serum (described previously in the Methods section) was added to the lytic-assay system. Addition of 0.1 ml of the antilysin serum resulted in 60% inhibition of the rate of lysis. Addition of 0.02 ml of antilysin serum resulted in only slight inhibition. Upon addition of 0.1-ml quantities of high-titer antiphage serum, no inhibition in rate of lysis was observed. The same lack of inhibition was also

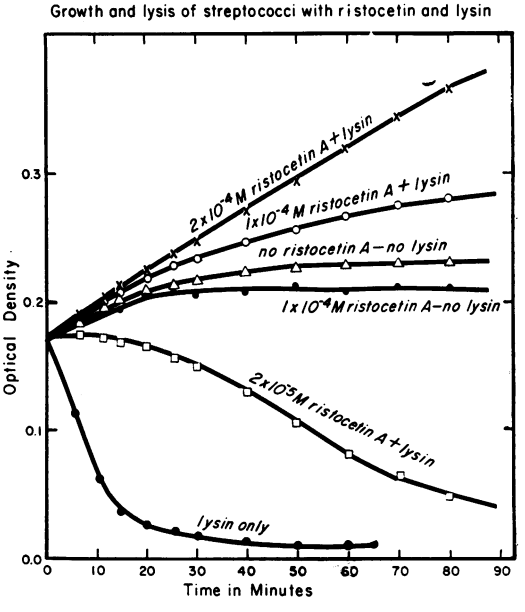

FIG. 9. *Growth and lysis of streptococci in the presence of ristocetin and lysin. Phosphate-buffered Todd-Hewitt assay medium used, and ristocetin added as indicated (0.1 ml crude lysin added).*

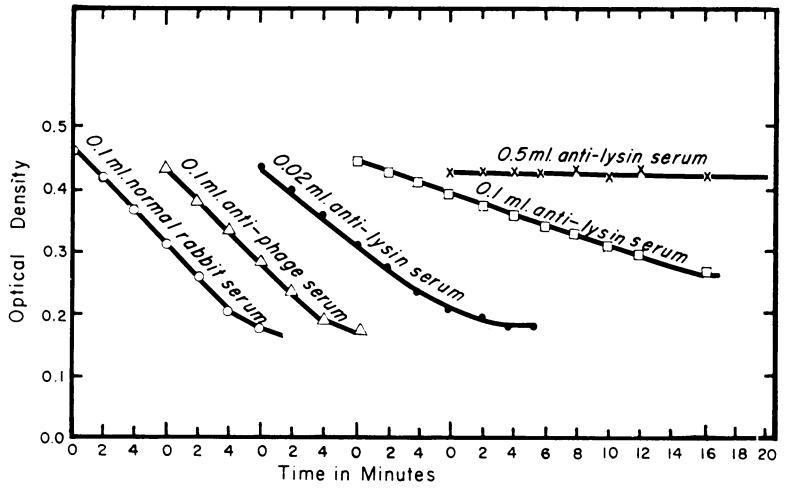

FIG. 10. *Lysis inhibition by specific antiserum. Streptococcal cells suspended in phosphate assay medium, additions of 0.05 ml of crude lysin and serum as indicated.*

observed when the phage antiserum content was increased tenfold. Normal rabbit serum showed no inhibitory effects on the lytic system for the above concentrations of sera. These results (Fig. 10) confirm that lysis is not due directly to the action of the phage, but to the lysin produced after infection of the group C streptococci with phage.

5) Effect of lysin on isolated cell walls:—When suspensions of cell walls are treated with large quantities of freshly prepared crude lysin, a decrease in turbidity occurs (Krause, 1957, 1958). Krause reported that this decrease in turbidity occurred when equal volumes of cell-wall suspension and lysin were used. In the work reported here, seven parts of lysin and three parts of cell suspension gave the results shown in Fig. 11. Equivalent rates of lysis of intact cells is obtained with less than 1 part crude lysin to 300 parts cell suspension, indicating that the rate of turbidity decrease for cell walls is less than 0.002% the rate of lysis of intact cells. Inspection of Fig. 11 also indicates that the decrease in turbidity of cell-wall suspensions is not complete, whereas intact cells gave complete clearing. The addition of EDTA or chlortetracycline to lysin-treated cell-wall suspensions did not increase the rate of lysis. This is in marked contrast to similarly treated whole-cell suspensions, where the addition of those two substances markedly increased lysis (Fig. 6 and 7).

Another marked difference in the behavior of

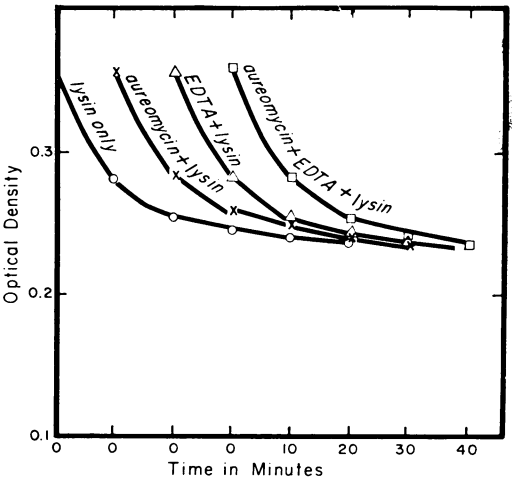

FIG. 11. *Turbidity decreases in cell-wall suspensions with lysin, EDTA, and chlortetracycline. Each tube contained 3.0 ml of S 23 cell walls in phosphate assay medium, and 7.0 ml freshly prepared crude lysin. The additions shown were 1.0 mg of chlortetracycline and 10^{-3} M EDTA.*

cell-wall suspensions is illustrated in Fig. 12. Lysis of whole-cell suspensions was markedly inhibited by *p*HMB, but there was no effect upon the rate of turbidity decrease of cell-wall suspensions by *p*HMB even at 10^{-3} M concentration. Similarly, although ristocetin A at a concentration of 5×10^{-5} M inhibits lysis of whole cells, it has no effect upon lysin action against isolated cell walls.

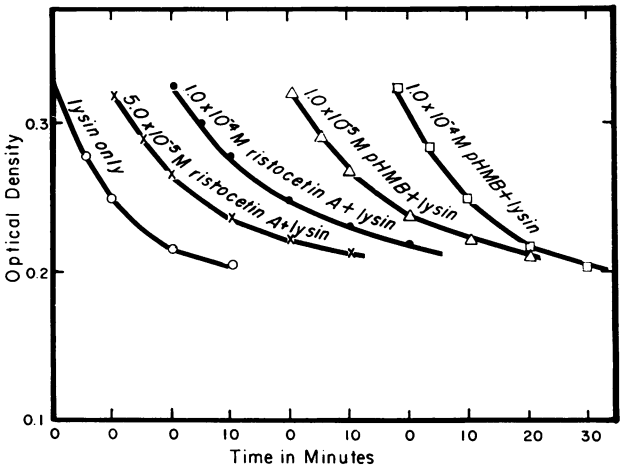

FIG. 12. *Turbidity decrease in cell-wall suspensions with lysin and various additions. Each tube contained 3.0 ml of S 23 walls in phosphate assay medium and 7.0 ml of freshly prepared crude lysin.*

These data indicate that the lysis of whole cells must, in part, be dependent upon enzymatic steps which are inhibited by the added substances; whereas, the lysis of cell-wall material is effected by enzymes which are not sensitive to these inhibitors.

DISCUSSION

This study of the phage-induced lysin has confirmed and extended the observations of Krause (1957, 1958). In Krause's purification of the phage-induced lysin, an ammonium sulfate precipitation was followed by 3 days of standing to precipitate the enzyme. We have found that such a long storage interval results in activity losses approaching 90%. With prior use of protamine sulfate to precipitate nucleic acids, ammonium sulfate will precipitate lysin activity in 10 min, with a recovery of 89% of the original activity. This modified procedure gives a 17-fold purification of lytic activity, with an over-all recovery of 48% of the original activity found in a crude lysate.

The lytic action is not due to the presence of the phage itself, but is produced by the infected group C streptococci at the time of lysis (Krause, 1958). We have shown that when antiserum for lysin is added to the lytic system, lysis is inhibited. On the other hand, the addition of anti-phage serum is without effect on lysis.

Although most of our investigations have dealt with the effects of different conditions upon the lytic process, it is clear that the nature and condition of the susceptible cells are also important. The lysin shows high and complete activity against group A streptococcal cells, lesser activity against group C cells, and, apparently, does not affect groups B and G streptococci. In addition, we have observed that detergents such as cetyltrimethylammonium bromide and neutralized deoxycholate, in concentrations of 10^{-5} M, markedly accelerate the rate of lysis of intact cells. The physiological age of cells also seems to be an important factor, since susceptibility to lysis decreases as cells stand at room temperature and is completely gone after 5 days of standing. It appears that cells must be capable of metabolism for the lysin to affect them.

The enzymatic nature of the lysin was indicated by Krause (1958) and further studied in these investigations. The lysin has an optimal pH between 6.0 and 6.7. A monovalent cation requirement by the lysin can be satisfied by Na^+, K^+, and Li^+. Divalent ions such as Mn^{++} and Ca^{++} do not satisfy the metallic ion requirement of the lysin, and there appears to be no anion requirement.

The lysis of intact cells appears to be dependent upon the presence of free sulfhydryl groups on the enzyme. The lysis is inhibited strongly by a 10^{-5} M concentration of *p*HMB, but the inhibition is readily reversed by the addition of cysteine. Strong reducing agents such as sodium hydrosulfite stimulate the rate of lysis, indicating, again, that free sulfydryl groups are probably

involved. The effect of *p*HMB seems to be exerted specifically upon the lysin. Prior incubation of cells with *p*HMB and subsequent exposure to lysin have no inhibiting effect on lysis. There is evidence, however, that the condition of the cell walls in intact organisms affects lysis. Low concentrations (10^{-5} M) of detergents such as neutralized deoxycholate and cetyltrimethylammonium bromide, which are known to affect the surface of the bacterial cell, also enhance lysis of cells in the system studied here.

Among the substances which were found to increase the rate of lysis are such diverse compounds as EDTA, potassium cyanide, chlortetracycline, streptomycin, and penicillin. These compounds enhanced the rate of lysis of cells in Todd-Hewitt broth, but had no appreciable effect when the same system was studied in potassium phosphate buffer. Since all of the above substances are known to bind metals to a greater or lesser degree, and lysis is immediately accelerated upon their addition, we have interpreted the above facts to indicate that Todd-Hewitt broth contains inhibitory substances, possibly trace metals.

The action of other antibiotics tested does not lend itself to easy explanation. Bacitracin A stimulates lysis but is not generally considered a metal-binding compound. It does, however, precipitate with heavy metal salts (Anker et al., 1948), so that there may be metal-binding involved in the bacitracin-stimulated lysis.

Another antibiotic, ristocetin A (10^{-5} M) had a markedly inhibitory effect upon lysis. In concentrations greater than 10^{-4} M, however, not only was lysis inhibited, but there was a marked increase in turbidity of the bacterial culture beyond that of a normal culture maintained under the same conditions. No explanation has been found yet as to the significance of this increased turbidity. Whether it is owing to an increase in size or an increase in number of bacteria is not known, but it is of interest to note that the higher levels of ristocetin are approaching substrate levels.

When isolated cell walls of group A streptococci were incubated with the lysin, a number of differences in behavior manifested themselves. The rate of lysis was much slower with cell walls, and approached the rate of lysis of whole cells only when massive amounts of lysin were added. Dissolution of the cell walls was not inhibited by *p*HMB or ristocetin A, in contrast to intact cells. Furthermore, there was no stimulation of cell-wall lysis by EDTA, chlortetracycline, streptomycin, or penicillin in Todd-Hewitt broth.

We have concluded, on the basis of the above evidence, that the lysis of whole cells is a multistep process. The lytic steps are susceptible to inhibition by metals, *p*HMB, and ristocetin A. The processes leading to the lysis of isolated cell walls, however, are not affected by the above reagents.

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