JOURNAL OF BACTERIOLOGY, Dec. 1968, p. 1895-1902
Vol. 96, No. 6

Bacteriocin (Hemolysin) of *Streptococcus zymogenes*

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Received for publication 18 September 1968

The sensitivity of *Streptococcus faecalis* (ATTC 8043) to *S. zymogenes* X-14 bacteriocin depends greatly on its physiological age. Sensitivity decreases from the mid-log phase on and is completely lost in the stationary phase. The sensitivity of erythrocytes to the hemolytic capacity of the bacteriocin showed considerable species variation. The order of increasing sensitivity was goose < sheep < dog < horse < human < rabbit. However, when red cell stromata were used as inhibitors of hemolysis in a standard system employing rabbit erythrocytes the order of increasing effectiveness was sheep < rabbit < human < horse < goose. When rabbit cells were used in varying concentrations with a constant hemolysin concentration, there was a lag of about 30 min, which for a given hemolysin preparation was constant for all red cell concentrations. Furthermore, the rate of hemolysis increased with increasing red cell concentration. If red cells are held constant and lysin varied, the time to reach half-maximal lysis varies directly with lysin but is not strictly proportional. Bacterial membranes were one to three orders of magnitude more effective than red cell stromata as inhibitors. The order of increasing effectiveness seems to be *Escherichia coli* < *Bacillus megaterium* < *S. faecalis* < *Micrococcus lysodeikticus*. In addition to membranes, a D-alanine containing glycerol teichoic acid, trypsin in high concentration, and deoxyribonuclease also inhibited hemolysis. Ribonuclease, D-alanine, L-alanine, DL-alanyl-DL-alanine, *N*-acetyl-D-alanine, *N*-acetyl-L-alanine did not inhibit hemolysis.

A variety of bacteriolytic substances produced by group D streptococci were reported in 1963 by Brock et al. (5). Simultaneously, Brock and Davie (4) reported on a hemolysin produced by a strain of *Streptococcus zymogenes* which they designate X-14. They have presented sound genetic evidence that the hemolytic activity and the bacteriocin (bacteriolytic) activity of X-14 are attributable to the same substance. In addition, Davie and Brock (7) have demonstrated that a ribitol teichoic acid, composed of ribitol phosphate, D-glucose, and D-alanine, associated with the cellular membrane of X-14 inhibits the lysin and prevents X-14 from killing itself. Removal of the teichoic acid from X-14 spheroplasts renders them susceptible to lysin. The inhibitory capacity of isolated teichoic acid depends upon the presence of the D-alanyl ester. This fact seems to be reflected in partially resistant mutants which contain smaller amounts of base labile D-alanine than wild-type X-14.

The bacteriocin or bacteriolytic activity extends to a large variety of gram-positive organisms but not to the gram-negative organisms, although few have been examined (5). In each of the major groups of gram-positive organisms, there are representatives that are resistant to X-14 lysin. Whether the resistance of these organisms is also due to a teichoic acid, or related substance, or not, has not been examined. However, the evidence presented by Davie and Brock (6) suggests that the group D bacteriocin (hemolysin) may be a general membrane-lytic agent and thus may be useful in examining membrane structure. Our interest in this substance derives from that possibility and from the further possibility that its inhibition by teichoic acid may be exploited to devise an assay for teichoic acid. These possibilities obviously require a more detailed understanding of the lytic nature of bacteriocin. In this paper, we report on some of our results.

MATERIALS AND METHODS

Strains. S. zymogenes X-14 was provided by T. D. Brock. *S. faecalis* (ATCC 8043) was obtained from the Syracuse University stock cultures. *E. coli* C600 was provided by R. Huskey. *M. lysodeikticus* 4698 was obtained from the American Type Culture Collection. *B. megaterium* KM was obtained from J.

[1] Predoctoral trainee of the National Institutes of Health (2 T0I GM00991-5).

Merrick. *S. pyogenes* 1-RP41 was obtained from R. Lancefield.

Media. Brain Heart Infusion Broth (BHI; Difco) was used exclusively as the growth medium for *S. zymogenes* X-14 and *S. faecalis* 8043. These strains were routinely carried on BHI agar plates containing 5% sheep erythrocytes. *E. coli* C600 was grown in Tryptone Broth (Difco), supplemented with 0.5% NaCl, 0.1% dextrose, and 10 μg of thiamine per ml. The final *p*H was adjusted to 7.5 with 1 M NaOH. *B. megaterium* was grown in Nutrient Broth (Difco). *M. lysodeikticus* was grown according to the method of Beers (1).

Phosphate-buffered saline. Phosphate-buffered saline (PBS) was used in two different concentrations. PBS-A contained 0.01 M phosphate with 0.145 M NaCl, and it was used for all preparations involving red cells. PBS-B contained 0.02 M phosphate with 0.085 M NaCl, and it was used for washing bacterial cells and membranes. The *p*H of both buffers was 6.8.

Lysin preparation. Crude lysin was isolated as the supernatant fluid from an X-14 culture incubated at 37 C with slow shaking. When the optical density of the culture reached 90 Klett units (no. 54 filter), the bacteria were removed by centrifugation for 20 min at 20,000 × *g*. The lysin was stored at 4 C until assayed. At this temperature, activity declines slowly but we used preparations less than 24 hr old.

Demonstration of lysis. For bacteriolysis, the indicator organism. *S. faecalis* 8043, was grown to 90 Klett units (no. 54 filter), harvested by centrifugation, washed in PBS-B, and suspended in this buffer to approximately 0.3 optical density unit at 540 nm. Lysin (1 ml) was added to 4 ml of 8043 suspension, and the loss of turbidity with time at 37 C was recorded.

For hemolysis, all types of red cells were washed three times in PBS-A and finally suspended at 2% by volume in PBS-A. This suspension was added, as needed, to give the required final concentration of red cells to a flask containing PBS-A. Crude lysin was added in the desired quantity, and release of hemoglobin with time at 37 C was measured at 541 nm. Hemolysis was also demonstrated on blood-agar plates containing 5% by volume of whole blood from the relevant source in BHI agar. Bacteria were then streaked on the plates and incubated at 37 C for 72 hr. The plates were then examined at 24, 36, 48, and 72 hr for lysis.

Standard hemolytic assay. Hemoglobin released from erythrocyte preparations was followed with time at 541 nm. A 30-ml mixture was used in which rabbit erythrocytes (0.12%) and lysin (20% by volume) were incubated at 37 C in PBS-A. Samples (3 ml) were taken at various times, and the unlysed cells were removed by centrifugation (3,000 × *g*, 2.5 min). The supernatant fluid was read for hemoglobin at 541 nm against a sample which contained no lysin. Care was taken to see that all components were at 37 C prior to addition to the assay flask. The activities of different preparations were compared on the basis of the time to reach half-maximal lysis (T_{50}).

Membrane preparation. M. lysodeikticus 4698 protoplast membranes were prepared according to the method of Ishikawa and Lehninger (8). *B. megaterium* protoplast membranes were prepared according to the method of Weibull and Bergstrom (15). *E. coli* spheroplasts were prepared according to the procedure of Birdsell and Cota-Robles (3). The *E. coli* spheroplasts were osmotically lysed by suspension in distilled water for 20 min, and the membranes were recovered by centrifugation at 48,000 × *g* for 30 min after the addition of 0.2 μg of deoxyribonuclease per ml. They were then washed twice in distilled water. *S. faecalis* 8043 membranes were prepared by suspending a twice-washed log-phase culture to 200 Klett units (no. 54 filter) in PBS-B. Trypsin (1 mg/ml) and lysozyme (200 μg/ml) were added to the suspension, and the suspension was stirred slowly at room temperature for 1 hr. The mixture was centrifuged at 1,000 × *g* for 10 min. The 1,000 × *g* supernatant liquid was centrifuged at 48,000 × *g* for 30 min; the membrane pellet was washed twice with distilled water. Erythrocyte stromata, except goose, were prepared from three times washed cells by suspension in 20 volumes of distilled water (4 C). After mixing, the suspension was allowed to stand for 10 min; then the stromata were recovered by centrifugation at 48,000 × *g* for 30 min and washed twice in distilled water. Goose erythrocytes must be broken by ultrasound. We used a Biosonik 20-kc instrument (Bronwill Scientific, Rochester, N.Y.). Washed cells were suspended at 10% by volume in distilled water at 15 C. They were then given five intermittent exposures of 1.5 min with the Biosonik power setting at 80. The stromata were then recovered as above.

All membrane preparations were stored at 4 C and were used within 48 hr.

Inhibition of hemolysis. Presumptive inhibitors were added in graded quantities to a standard hemolytic assay mixture. These were brought to 37 C and added to a flask containing red cells at 37 C. The lysin at 37 C was added immediately after the "inhibitor." The time of lysin addition was taken as zero-time. Control flasks containing red cells and inhibitor but no lysin and others containing red cells and lysin but no inhibitor were included.

Membrane preparations were washed not less than three times in distilled water and finally centrifuged at 2,400 × *g* prior to addition. Enzymes (deoxyribonuclease, ribonuclease, and trypsin) were preincubated with crude lysin for 1 hr at 37 C then were added without further treatment to assay flasks. *S. pyogenes* 1-RP41 teichoic acid was added with and without preincubation with lysin.

Chemicals. Both deoxyribonuclease (B grade, deoxyribonuclease 1) and ribonuclease (five times crystallized, A grade) were obtained from Calbiochem, Los Angeles, Calif. Both were from bovine pancreas. Lysozyme (twice crystallized, code LY) and trypsin (once crystallized, 2,900 National Formulary units/mg) were obtained from Worthington Biochemical Corp., Freehold, N.J. Teichoic acid from *S. pyogenes* 1-RP41 was extracted by using the phenol method of Moskowitz (13).

RESULTS

The lysin is effective against both erythrocytes and a wide number of gram-positive organisms. Brock and Davie (4) took advantage of its hemolytic properties and devised an assay which measured the loss of turbidity of an erythrocyte suspension. This approach did not suit our purposes, so we initially examined bacteriolysis by using a sensitive strain of *S. fecalis* ATTC 8043. The sensitivity of 8043 is very much dependent on its physiological state. This strain becomes increasingly resistant to lysin from approximately the mid-log phase on (in BHI medium); it becomes completely resistant in the stationary phase. When 8043 cells were grown to 90 Klett units (no. 54 filter), harvested, washed, and then suspended in PBS-B at an optical density of approximately 0.280 at 540 nm, there was, following addition of lysin, a delay before a decline in turbidity was evident. This lag was observed to vary between 15 and 45 min with different preparations. Turbidity declined in a linear fashion during the next 90 min, but the rate varied with individual lysin preparations. In the absence of lysin, no decrease in the turbidity of these log-phase cells was ever observed. Furthermore, stationary cells, washed and suspended with lysin in the same manner, also never exhibited a decline in turbidity.

To examine the hemolytic effect of lysin, we first observed the relative sensitivities of a variety of erythrocyte types.

In a limited number of tests, the effects of lysin on human erythrocytes of A, AB, B, and O blood types were examined. No significant differences in susceptibility to the lysin among these types were found except in one case in which the red cells of an A individual were significantly more susceptible than all others. The possibility that A_1 and A_2 blood types may show different susceptibilities to lysin was not examined.

The results (Fig. 1) were typical of those obtained when red cells from sheep, horses, rabbits, dogs, and geese were compared for susceptibility to lysin. That there are species differences was very clear. Of the mammalian species, rabbits erythrocytes were most easily lysed, whereas sheep seemed to be the most resistant. Goose erythrocytes appeared to be completely resistant in assays performed in flasks, but when they were incorporated in blood-agar plates, lysis was evident in about 24 hr. It should be noted that goose red cells are resistant to lysis by most means and are usually broken up by means of ultrasound.

Since rabbit red cells are most readily lysed, we elected to examine them in detail because they

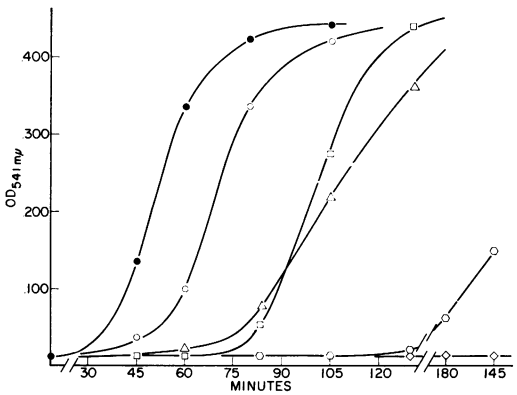

FIG. 1. *Relative sensitivities of erythrocytes to crude X-14 lysin. Symbols:* ●, *rabbit cells;* ○, *human cells;* □, *horse cells;* △, *dog cells;* ⬡, *sheep cells;* ◇, *goose cells. All cells were added to 0.12% based on packed cell volume with a constant lysin concentration.*

would provide an assay with greater sensitivity. Figure 2A shows the results of varying erythrocyte concentration and of measuring the release of hemoglobin with time when crude culture filtrate was used as a source of lysin. A group of sigmoidal curves was produced; such curves are typical of hemolysis (11). For all red cell concentrations, there was a delay of approximately 30 min before a detectable quantity of hemoglobin was released. When the concentrations were between about 0.08 and 0.5%, the time to reach half-maximal lysis (T_{50}) was essentially constant. At these concentrations, the T_{50} became a characteristic for a given crude culture filtrate. Although it is not clearly shown in Fig. 2A, we have, on repeated occasions, received the impression that at red cell concentrations below approximately 0.08% there is a definite tendency for the T_{50} to decrease with decreasing cell concentration. At about 0.025% concentration, absorption by the lysin preparation becomes significant, and thus no conclusion can be drawn about this tendency. The "linear" portions about the inflection point of the sigmoidal curves are presented in Fig. 2B. In this graph, the slopes of the curves are continuously increasing with increasing cell concentration throughout the entire range employed. Thus, the apparent rate of hemolysis must be increasing with increasing red cell concentration.

The results in Fig. 3 were obtained by using a red cell concentration of 0.12% and by varying the volume of crude culture filtrate added. As before, a group of sigmoidal curves was produced. In this case, the delay before release of hemoglobin was observed to decrease with increasing

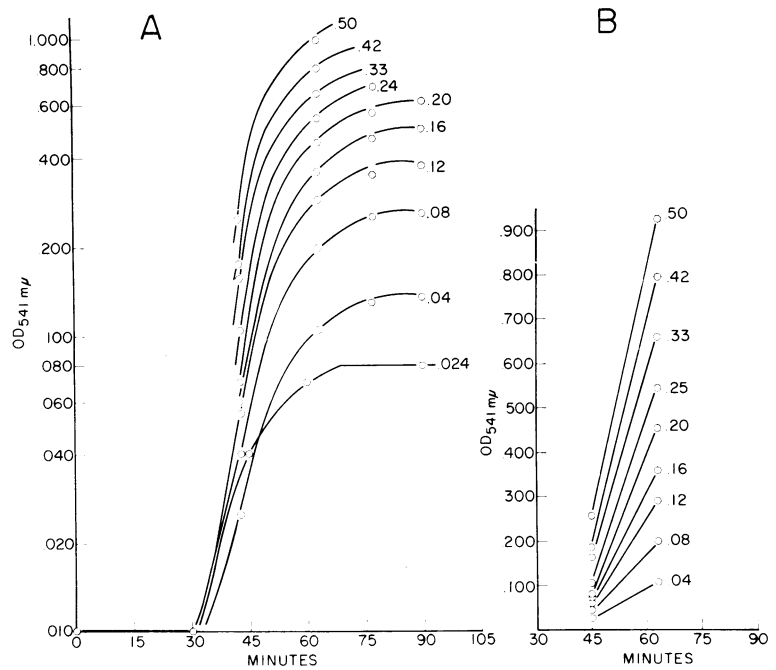

FIG. 2. *(A) Hemoglobin release with constant crude lysin and varying rabbit erythrocytes. Numbers indicate volume per cent red cells based on packed cell volume of washed cells. (B) Slopes of inflection point regions of curves in A.*

lysin concentration and tended toward an irreducible length of time. The T_{50} also decreased with increasing lysin within the concentration limits reported. If T_{50} is graphed as a function of lysin added, the result is curvilinear. The function approaches linearity provided the quantities added are sufficiently large. When too small quantities are added, total lysis finally occurs, but the time required is so long that browning of the hemoglobin occurs, rendering the readings at 541 nm nonlinear.

We devised, for routine use, an assay for lysin based on the release of hemoglobin from rabbit erythrocytes in accordance with the data presented above. In this assay, 0.12% rabbit red cells were used as "substrate," and the release of hemoglobin with time was followed. Lysin activities were compared on the basis of T_{50} values. With this method, it was necessary to use lysin quantities that would give T_{50} values of less than 150 min. In practice, this meant adding at least 4 ml of crude culture filtrate to the assay mixture which has a total volume of 30 ml.

To demonstrate inhibition of lysis, suspected inhibitors were added to the standard assay system, and the increased time for lysis to complete its course was observed. When the stromata

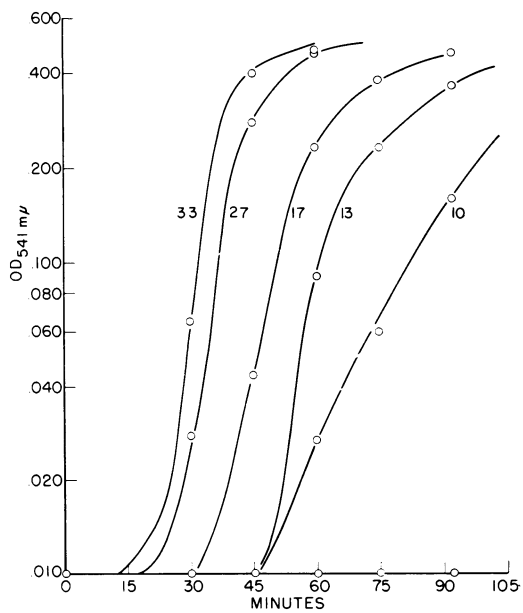

FIG. 3. *Hemoglobin release with 0.12% rabbit erythrocytes and varying crude lysin. The numbers indicate volume percent of culture filtrate in assay mixture.*

from laked rabbit erythrocytes were added in graded quantities to a series of standard assay mixtures, a series of curves resulted (Fig. 4). Here, the usual sigmoidal curves were displaced in time. Furthermore, the slopes of the curves decreased with increasing quantities of stromata.

In a similar manner, the stromata of different red cell types were compared. The order of effectiveness as inhibitors was goose > horse > human > rabbit > sheep (Fig. 5). This is not the order of susceptibility to lysis. In fact, with the exception of sheep, it is the reverse of that order.

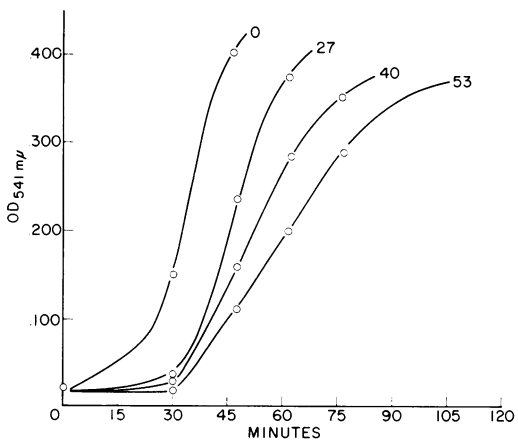

FIG. 4. *Inhibition of hemoglobin release by rabbit red cell stromata. Numbers indicate final concentration (μg/ml) of membrane protein in the standard hemolytic assay mixture.*

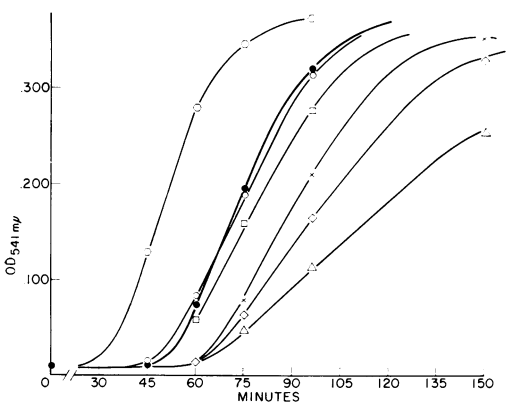

FIG. 5. *Relative inhibition of hemoglobin release by different red cell stromata. The final concentration of membrane protein is in each case 83 μg/ml of stromata, added to the standard assay mixture. Symbols: ⬡, dog stromata; ○, sheep stromata; □, rabbit stromata; ×, human stromata; ◇, horse stromata; △, goose stromata; ●, no stromata.*

Curiously, dog stromata decreased rather than increased the time to reach half-maximal lysis.

We also examined various bacterial membrane preparations for their ability to inhibit hemolysis. As with red cell stromata, when membranes of *M. lysodeikticus* were added in graded amounts to a series of standard assay mixtures, the hemoglobin release curves were displaced in time, and the slopes of the curves decreased with increasing membrane concentration (Fig. 6). To compare the relative inhibitory capacities of the membranes, the quantities of membranes required to cause the T_{50} to double are presented in Table 1. Clearly, bacterial membranes were much more effective than stromata, although they too showed a wide variation in effectiveness as inhibitors.

In addition to the membrane preparations, we examined various other substances for inhibitory capacity. We had previously observed that *S. pyogenes* 1-RP41 was resistant to lysis by X-14 lysin. This group A streptococcus contains an "intracellular" glycerol teichoic acid in contrast to the ribitol teichoic acid of *S. zymogenes* X-14. The glycerol teichoic acid is reported to be composed solely of glycerol phosphate and D-alanine (9). Glycerol teichoic acid was extracted by using phenol (13), and its ability to inhibit hemolysis was examined. It was an effective inhibitor (Fig. 7). Approximately 6 to 7

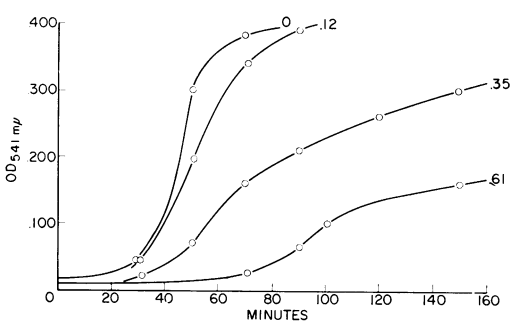

FIG. 6. *Inhibition of hemoglobin release by M. lysodeikticus membranes. Numbers indicate final concentration (μg/ml) of membrane protein in the standard assay mixture.*

TABLE 1. *Comparison of inhibitory capacities of membranes from various sources*

Source	Membrane protein required to double T_{60}
	μg/ml
M. lysodeikticus 4698	0.24
S. faecalis 8043	1.2
B. megaterium KM	17
Erythrocytes	100–300

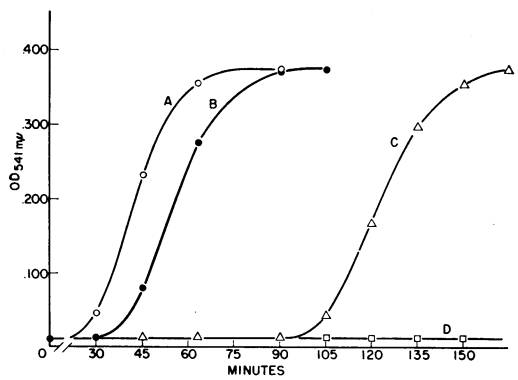

FIG. 7. *Effects of S. pyogenes 1-RP41 teichoic acid on hemoglobin release. Curve B: standard assay mixture. Curve A: 3.3 μg/ml polyglycerophosphate in standard assay. Curve C: 6.7 μg/ml D-alanyl-polyglycerophosphate. Curve D: 13 μg/ml D-alanyl-polyglycerophosphate.*

μg/ml on a weight basis gave a demonstrable inhibition, whereas 13 μg/ml completely inhibited hemolysis. If D-alanine is removed by mild base hydrolysis (NaOH, *p*H 11.5, 240 min at 37 C), then not only is the inhibitory capacity lost, but a significant enhancement of hemolysis was observed.

It is known that the 1-RP41 teichoic acid binds to red cells (9) and that D-alanine apparently is necessary to this binding. It was of interest, therefore, to observe if red cells preincubated with 1-RP41 teichoic acid would be protected from lysin. Accordingly, 2.0% red cells were incubated with an equal volume of 1-RP41 extract in PBS-A (*p*H 6.8) for 30 min at 37 C, washed three times with PBS-A, and suspended to 0.12% in a standard assay mixture. The 1-RP41 extract contained approximately 200 μg of teichoic acid per ml. It was observed that preincubated cells were, in fact, not protected but were rendered more sensitive to lysin than normal cells.

We also tried to inhibit hemolysis by adding to the standard assay system the following: D-alanine, DL-alanyl-DL-alanine, *N*-acetyl-D-alanine, *N*-acetyl-L-alanine, and L-alanine. All were added to give a concentration of 1 mg/ml. None of these exhibited the capacity to inhibit hemolysis at this concentration.

In another approach, the lysin preparations were preincubated with deoxyribonuclease (10 μg/ml), ribonuclease (57 μg/ml), and trypsin (143 μg/ml) for 1 hr at 37 C; then the treated preparation was added to a standard hemolytic assay system. Ribonuclease had no effect, trypsin showed a small but significant inhibition, but deoxyribonuclease was quite effective as an inhibitor. At a higher concentration of deoxyribonuclease, (57 μg/ml), inhibition of hemolysis after pretreatment of the lysin appeared to be complete. That inhibition by deoxyribonuclease was due to some property other than its nuclease activity is suggested by the fact that in 10^{-3} M ethylenediaminetetraacetate (EDTA) deoxyribonuclease was still fully effective as a hemolysis inhibitor. However, the nuclease activity of deoxyribonuclease has an absolute requirement for Mg^{++}, and at 10^{-3} M EDTA, the nuclease activity is completely inhibited.

DISCUSSION

The increasing resistance of *S. faecalis* 8043 with increasing age of the culture is reminiscent of the observation by Shockman et al. (13) that *S. faecalis* 9790 is susceptible to its own autolysin only at a particular physiological age. It is not possible to unequivocally rule out the notion that an autolysin participates in bacteriolysis subsequent to attack by group D lysin. However, if an autolysin is involved, it must differ in some degree from the one reported by Shockman. From their data with 9790, one might expect the turbidity of a suspension of washed log-phase 8043 cells at 37 C to decline very significantly in under 60 min without the intervention of an external agent. However, our controls showed no decline whatever in more than 3 hr at 37 C.

It is tempting to speculate that resistance to lysin in 8043 results from a teichoic acid which is synthesized in late log phase. However, this point has still to be examined.

An assay for lysin using 8043 as an indicator could have been devised. However, turbidity measurements are not especially sensitive; but more important, it is necessary to very rigidly control the physiological state of this organism to obtain reproducible results. Thus, for routine use, bacteriolysis is unsuitable, although it was, in fact, used for certain purposes.

The general picture obtained for hemolysis by group D lysin is similar to that reported for other hemolysins (2, 11). When red cell concentrations were varied, the lag period was essentially constant for all red cell concentrations, and the time required for completion of hemoglobin release was also constant for all concentrations (Fig. 2). When lysin was varied with a constant red cell concentration (Fig. 3), the lag period decreased with increasing lysin but tended toward an irreducible time when lysin concentration became very large.

Thus, it can be concluded that the length of the lag period depends upon the quantity of lysin present, and within the limits used, is independent

of red cell concentration. Similarly, the time to reach half-maximal lysis (T_{50}) is also independent of red cell concentration. Hence, both the lag time and T_{50} are characteristics of a given lysin preparation. When both of these are graphed as functions of lysin concentration, the result is curvilinear.

Our results and the models we constructed therefrom are so closely similar to those reported by Marucci (11, 12) for staphylococcal α-toxin that nothing would be added to the literature by further discussion in this paper. We did not make a systematic examination of bacteriolysis, but the general pattern of the results that we do have indicates a similar picture to that of hemolysis.

It cannot be unequivocally stated that lysin is an enzyme. Final proof rests on finding a defined substrate. However, it seems probable that lysin is an enzyme for at least two reasons. (i) It seems highly unlikely that a nonenzymatic substance would have the specificity implied by the inhibition of lysin by D-alanyl esters of teichoic acid and by possibly sterically similar lecithin (6). (ii) We know that lysin is composed of at least two inactive subunits, which in combination are lytic. However, only one of these contains the "active site" (*unpublished data*). It would be an unusual nonenzymatic substance that behaved in this way.

The goal of devising an assay for teichoic acid rests on having a reliable assay for lysin. As noted above, the T_{50} values (Fig. 3) are curvilinear when graphed as a function of lysin concentration. The change in rates of hemolysis, therefore, is not directly proportional to the lysin concentration. However, the deviation from linearity is small if lysin represents at least 12 to 15% of the assay volume using the preparations obtained from standard growth conditions. Hence, lysin can reliably be assayed in this manner knowing that a systematic error is being incorporated. In practice, any preparation which gives a T_{50} greater than 150 min is discarded because, beyond this, the deviation from linearity becomes uncomfortably large.

To use inhibition of hemolysis as an assay of inhibitor incorporates an added error, because the inhibitor changes the system in a way which cannot yet be precisely analyzed. The changes in slopes (Fig. 4 and 6) probably are due to a continuously changing ratio of available lysin to the red cells. Nevertheless, the general tendency is clear and predictable, and measuring the inhibitor by degree of inhibition gives values that are at least crudely quantitative. However, one further caution must be raised about our ordering of membranes with respect to inhibition capacities. Masses are compared by measuring protein content, but this may not give a reliable comparison for any property not related to protein content. At this time, it cannot be said whether this is true for lysin activity or not. Nevertheless, the order of bacterial membranes is, perhaps, close to what would be expected on the basis of degree of relatedness of the organisms.

Inhibition of hemolysis obviously can occur in two distinct ways: by inhibiting lysin or by changing the surface of the red cells. These two modes of action cannot in every case be readily distinguished. Preincubation of red cells with membranes does not seem to alter their sensitivity to lysin.

Inhibition by membrane preparations may not be inhibition in the strict enzymological sense, since presumably only additional substrate is being added. Although the membrane preparations are washed, quantities of teichoic acid, deoxyribonuclease, or other unknown inhibitors may also be included. No systematic attempt was made to rule out these possibilities, since we believe that in sum the data present a reasonable case that the major cause of inhibition is some structural component common to all the membranes. Furthermore, we know that lysin has a direct effect on isolated *M. lysodeikticus* membranes. The nature of this effect is now under study.

Trypsin inhibition of hemolysis occurs only with concentrations that are much higher than normally used for proteolysis. At these concentrations, there may be an effect on the red cell rather than on lysin. At this time, the trypsin effect has not been examined in detail. Inhibition by deoxyribonuclease, on the other hand, cannot readily be explained by its known properties. Certainly inhibition by deoxyribonuclease is not caused by its nuclease activity since that requires Mg^{++}, and inhibition of lysis is unaffected by 10^{-3} M EDTA. Two other possibilities have occurred to us. Lysin contains a component that resembles a nucleic acid which binds deoxyribonuclease, or deoxyribonuclease, since it is from pancreas, contains proteolytic enzymes that degrade lysin. With respect to the latter, the effect of our deoxyribonuclease preparations on anthranilic synthetase was examined. It had no apparent effect but that, of course, is inconclusive. In regard to the former, we have some highly incomplete data which suggest that a component of lysin might actually be a teichoic acid. Should this be true, then it is conceivable that deoxyribonuclease binds to that component rendering lysin inactive. It should be pointed out that pancreatic deoxyribonuclease was used in preparing all bacterial membranes, except in the

case of *S. faecalis*. However, since the quantities added are very small and the membranes are washed, it seems unlikely that inhibition derives from this source.

Inhibition of hemolysis by glycerol teichoic acid from *S. pyogenes* 1-RP41 follows our predictions and indicates that lysin may be inhibited by most teichoic acids bearing a D-alanyl ester.

Our data and those of earlier investigators suggest that X-14 lysin may be a general membrane-lytic agent. If this is true, one wonders why an organism would possess such a substance and why does it release it to the medium? Brock and Davie (4) have suggested that the lysin confers an ecological advantage on X-14 allowing it to displace other species. We have no doubt that this is true, but we think it important to note that nature, in evolution, frequently uses old machinery for new work rather than building new machinery. Three phenomena seem important in this context: (i) the possibly general membrane-lytic capacity of X-14 lysin, (ii) the widespread occurrence of similar lysins in bacteria, and (iii) the antagonism of one normal component by another normal component in the same cell. These lead us to speculate that X-14 lysin and, perhaps, all bacterial lysins play some role in the biosynthesis of membranes. This role, whatever it may be, is moderated by synthesis of teichoic acid or a similar substance. Release of the lysin may be accidental or it may be one way of getting rid of a material that, having fulfilled its function, becomes potentially deleterious.

ACKNOWLEDGMENTS

This investigation was supported by grant GB-4900 from the National Science Foundation and by Public Health Service research grant 1 R01 A107361-01 from the National Institute of Allergy and Infectious Diseases.

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