LYSIS OF BRUCELLAE BY THE COMBINED ACTION OF GLYCINE AND A LYSOZYME-LIKE AGENT FROM RABBIT MONOCYTES

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ABSTRACT

Ralston, Doris J. (University of California, Berkeley), B. S. Baer, and S. S. Elberg. Lysis of brucellae by the combined action of glycine and a lysozyme-like agent from rabbit monocytes. J. Bacteriol. 82:342-353. 1961.—An acid-extractable lytic material was obtained from rabbit monocytes. It acts on a substrate in the walls of brucellae and has properties similar to egg-white lysozyme. Brucella melitensis strain Rev 1s is completely resistant to this agent and also to crystalline lysozyme, but when the cells are exposed to sufficient amounts of glycine, the surface is rendered susceptible to these lytic agents. Rough type Rev Is are more susceptible than smooth, and the virulent B. melitensis strain 6015 is most resistant.

Since the studies of Metchnikoff (1907) phagocytic cells have been examined for their content of bactericidal, growth-inhibitory, and lytic materials. A number of agents have been described, including the leukins (Schneider, 1909), phagocytin (Hirsch, 1956a, b), leucocytes A, B, and C (Amano et al., 1954, 1955, 1956), a lipo-protein fraction from rat polymorphonuclear mitochondrial fractions (Fishman and Silverman, 1957), and the well-known lysozymes of tissues and fluids (Fleming, 1922). According to Skarnes and Watson (1957) most of these antibacterial factors have been identified as basic proteins, polypeptides, or polynamines. In general they appear to be more active against gram-positive than gram-negative bacteria.

The persistence of brucellae within tissue cells and large mononuclear phagocytes of infected hosts has focused interest upon cellular activities supporting or limiting their growth. This paper describes a lysozyme-like agent from rabbit monocytes, which is capable of acting on a substrate in brucella walls. Equivalent amounts of this material have also been obtained from rabbits previously immunized with the vaccine strain of Brucella melitensis, Rev Is. When used alone, the agent does not affect living microorganisms, but combined with glycine, it causes an accelerated lysis and death. Experiments in tissue culture (Ralston and Elberg, 1960) indicate that glycine added to parasitized monocytes affects the intracellular multiplication of B. melitensis Rev Is. In the present studies, the differences in susceptibility of rough and smooth forms to the glycine-extract treatment seem to be correlated with the observed differences in their intramonicotylic multiplication.

MATERIALS AND METHODS

Bacterial strains. Rough and smooth types of the vaccine strain of B. melitensis Rev Is and virulent B. melitensis strain 6015 were maintained on Albin crystals brucella agar. The smooth or rough characteristics of the cultures were determined by oblique light inspection of colony growth, observation of gentian violet flooded plates and, on occasion, by the acriflavin agglutination technique (Henry, 1933; Braun and Bone, 1947; White and Wilson, 1951). The Rev Is strain (also referred to as Rev 1) was isolated as a streptomycin-independent reversion from an avirulent, streptomycin-dependent mutant of B. melitensis strain 6015 (Herzberg and Elberg, 1953; Elberg and Faunce, 1957). Its potentialities for use as a living, attenuated vaccine strain have been under investigation for several years (Elberg and Meyer, 1958). Because cells of this strain appear to be rapidly cleared from animal organs and tissues, it has been selected as a possible sensitive indicator for antibrucella factors which might be extracted from various tissue cells. On agar the Rev Is strain forms smaller colonies and develops more slowly than the more virulent strain 6015.

Preparation of rabbit monocyte exudates. Peritoneal exudates were collected on the fifth or sixth day after injection of 30 to 50 ml sterile
Klearol (L. H. Butcher Company, San Francisco) into 5-lb rabbits. The animals were killed with Beuthanasia (H. C. Burns Company, Oakland, Calif.) injected intravenously, 0.5 ml per rabbit. The peritoneal cavities were washed with 200 ml chilled saline or with Tyrode’s solution at pH 7.0 to 7.4 and the cell suspensions were aspirated through sterile cheesecloth into a separatory funnel. The residual oil was removed and the cells were centrifuged at 250 × g for 10 min, washed in 25 ml fresh solution, examined for cell composition, and then stored in the cold until extracted. Exudates which contained large numbers of red blood cells were discarded.

**Preparation of crude extracts.** The cell suspensions from the peritoneal exudates contained over 85% large mononuclear cells in total yields of from 1 × 10⁶ to 5 × 10⁶ cells per animal. The following preparations contained material which could be shown to have activity against brucella: (i) Exudates containing 1 × 10⁶ to 3 × 10⁶ monocytes per ml, harvested in saline or in Tyrode’s solution, washed within a few minutes in 25 ml saline, and resuspended in 5 to 10 ml of a solution composed of equal parts 1 N acetic acid and 0.85% saline, pH 3.5. The suspensions were frozen in Dry Ice and acetone and thawed at 28 C six to eight times, centrifuged to remove debris, neutralized with 10 N NaOH added dropwise with rapid stirring, and recentrifuged. On occasion, cell suspensions were treated in Tyrode’s solution with 100 μg per ml sterile trypsin for 1 hr at room temperature; then the residual cells were washed and extracted. (ii) Exudates harvested in an intracellular salts solution according to the procedure of Hirsch (1956a, b), washed, frozen, and thawed three times at pH 5.6, centrifuged, and neutralized. (iii) Exudates harvested and washed in Tyrode’s solution, resuspended in 10 to 20 ml of the same fluid, and stored at 4 C for 1 to 4 weeks prior to freeze-thaw treatment of the residual cells and debris.

**Enzymes, buffers, and test media.** Crystalline trypsin, deoxyribonuclease, ribonuclease, egg-white lysozyme, and D-amino oxidase were obtained from Worthington, pancreatic lipase from Nutritional Biochemicals, and purified chymotrypsin through the courtesy of John H. Northrop. Glycerine-HCl was prepared as a 3 M solution neutralized with 10 N NaOH.

The following media and buffers have been used to test the action of the monocyte agent and known enzymes on growth and lysis of brucella: (i) Albimi brucella broth; (ii) Tyrode’s-serum medium, designated TS, composed of 60% Tyrode’s solution and 40% unheated normal rabbit serum; (iii) 0.067 M and 0.01 M phosphate buffer alone or supplemented with Tyrode’s solution at pH 6.2 to 8.0.

**Preparation of cells.** For preparing heated cells, smooth growth of Rev Is was harvested after 72 hr on Albimi brucella agar in ZoBell’s buffer (ZoBell and ZoBell, 1932). The cells were washed, resuspended to contain 10¹ⁱ organisms per ml in a volume of 250 ml, and heated at 60 C for 1 hr. For butanol extraction, the heated cells were centrifuged and resuspended in an equal volume of n-butanol for 24 hr at 4 C. The solvent was then decanted and the cells were washed and resuspended in saline. For tests of living cells, Roux bottles containing Albimi brucella agar were inoculated with 3-day growth of each strain. The inoculum was from an agar slant, suspended in 10 ml saline per slant, and dispensed in 2-ml portions per bottle. The cultures were incubated for given periods at 37 C. In general, only relatively young cells proved to be susceptible to the concentrations of glycerine and monocyte agent used in these experiments.

**Preparation of cell walls.** Cell walls of strain Rev Is and Staphylococcus aureus strain K₁ were prepared according to the procedure of Hancock and Park (1958). In this method the cells were extracted with 5% trichloroacetic acid at 90 C, washed, and exposed to 50 μg trypsin per ml at pH 8.0 until no further turbidity decrease occurred. Cells of strain Rev Is lost 80% of their initial turbidity and were difficult to centrifuge down. Microscopic examination showed that they stained only faintly gram-negative but still retained their typical coco-bacillary shape. They could be seen to better advantage when stained with Alcian Blue, a stain which combines with the mucopolysaccharides of the bacterial wall. Ultraviolet absorption readings of the supernatant fraction from each extraction step revealed the presence of considerable material absorbing at 260 and 280 μμ. These observations suggest that the chemically extracted residues represented cell walls. S. aureus treated by this procedure yielded cell walls of a purity similar to those of Hancock and Park.


Lysozyme tests. Amounts of 5 to 6 ml of cells in buffer were dispensed into standardized Klett tubes, mixed with enzyme, enzyme plus glycine, or appropriate control solutions, and incubated at 37 C. Turbidimetric readings were made with a Klett-Summerson photoelectric colorimeter equipped with a 660 mp filter. Over the range from 10 to 150 units, the readings were proportional to the bacterial count, a reading of Klett 20 being equivalent to approximately $6 \times 10^8$ cells per ml. The initial test turbidity was generally 50 to 100 Klett units. The percentage of initial turbidity remaining in each tube, i.e., the percentage of unlysed cells, was calculated from:

$\frac{\text{Klett units at time, } t}{\text{Klett units at time } 0} \times 100$

Curves were constructed showing the course of lysis at 37 C. As indicated in Fig. 1, the rate was not linear. The curves in Fig. 3 were plotted as the logarithms of the percentage of unlysed cells for each time interval, and these indicated that with either the monocyte material or egg-white lysozyme the lysis of Micrococcus lysodeikticus followed a logarithmic course, whereas that of the brucellae was more complex.

For assay of the equivalent lysozyme activity of the material in the monocyte, the extract was diluted in distilled water. Five-tenths milliliter of an appropriate dilution was mixed with 4.5 ml 0.02 m phosphate buffer and 0.5 ml of a 24-hr saline-washed suspension of M. lysodeikticus. The tubes were placed at 37 C and read at intervals. Known amounts of egg-white lysozyme were treated similarly. A standard curve was constructed representing the percentage of lysis in a given time for each amount of lysozyme. The equivalent activities of known dilutions of the monocyte extract were then determined from the graph. From these data the equivalent lysozyme activity per ml of packed cell button could be calculated. For example, in a typical assay, 0.1 ml packed button yielded 5.5 ml extract containing 23 $\mu g$ per ml lysozyme activity, a total of 126.5 $\mu g$ per 0.1 ml button or 1265 $\mu g$ per ml packed cells.

RESULTS

Lysozyme activity of monocyte preparations. There have been conflicting reports as to the lysin content of various type of phagocytes obtained from different exudates (Kling, 1910; Gengou, 1921; Amano et al., 1954; Myrvik and Weiser, 1955; and Ralston and Elberg, 1960). Myrvik and Weiser found less than 10 $\mu g$ per ml packed monocytes in Bayol F-induced peritoneal exudates. On the other hand, lung exudates high in monocytes and various exudates containing polymorphonuclear cells have been found to contain lysozyme activity equivalent to 1,000 to 3,000 $\mu g$ per ml packed cell button (Hirsch, 1960; Myrvik, 1960). In the present experiments, oil-induced peritoneal exudates high in monocyte-type cells have been found to contain large amounts of material lytic for M. lysodeikticus. Despite the high concentration of monocytes, these existed the possibility that the lytic activity was derived from polymorphonuclear cells or from lymphocytes. However, suspensions from exudates selected because of their relatively low polymorphonuclear count (less than 1%) were found to contain greater than 1,000 $\mu g$ per ml packed cells, and since lymphocytes from the mesenteric nodes were inactive, this suggested that the major source of the active material was the mononuclear phagocyte.

It was found that lytic activity was greatly inhibited when the tests were performed in buffers containing physiological saline or Tyrode’s solution. Under these conditions, egg-white lysozyme activity was not greatly affected. It is possible that discrepancies in the literature have been based upon differences in test conditions.

Action on killed brucellae and cell walls. Brucellae heated at 60 C for 1 to 2 hr and extracted with butanol, chloroform, or acetone became susceptible to the agent in the extracts. As indicated in Table 1, the cells underwent a loss in turbidity and a release of a viscous material identified as deoxyribonucleic acid. Egg-white lysozyme produced a similar effect. Trypsin, chymotrypsin, and lipase reduced the turbidity but failed to release any viscous material unless the cells had first been exposed to lysozyme or monocyte extract. When the enzyme-treated cells were washed out of the reaction mixture and resuspended in distilled water they did not undergo further lysis.

Figure 1 shows the lysis of brucella and S. aureus walls by egg-white lysozyme, the monocyte agent, and by a lysozyme-like enzyme from S. aureus, violysin (Ralston et al., 1955). The monocyte agent and lysozyme lysed both bacterial preparations but violysin acted only on S. aureus, indicating that the violysin substrate was absent from the brucella wall.
TABLE 1. Effect of enzymes on heated, butanol-treated Rev Is in 0.067 M phosphate buffer, at pH 7.5

<table>
<thead>
<tr>
<th>Suspending medium</th>
<th>Enzyme</th>
<th>Untreated cells*</th>
<th>Residual cells after lysozyme treatment</th>
<th>Residual cells after extract treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>% Lysis</td>
<td>Viscosity</td>
<td>% Lysis</td>
</tr>
<tr>
<td>Distilled water</td>
<td>Lysozyme</td>
<td>20</td>
<td>+</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Extract</td>
<td>16</td>
<td>+</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Trypsin</td>
<td>30</td>
<td>0</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>Chymotrypsin</td>
<td>18</td>
<td>0</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>Lipase</td>
<td>16</td>
<td>0</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>Papain</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>d-Amino oxidase</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Deoxyribonuclease</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Ribonuclease</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Buffer</td>
<td>Extract</td>
<td>16</td>
<td>0</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>Lysozyme</td>
<td>20</td>
<td>+</td>
<td>0</td>
</tr>
</tbody>
</table>

* Initial turbidity of cells corresponded to 100 Klett units. All test cultures for lysis were incubated at 37°C until no further drop in turbidity occurred.

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**Fig. 1.** Comparative action of egg-white lysozyme, monocyte extract, and a phage-induced staphylococcal lysis on brucella and staphylococcal walls.
It was noted that the monocyte extract exhibited greater activity for brucellae than for S. aureus, whereas with lysozyme, the ratio was reversed, perhaps indicating a difference between the two agents.

Action on living and glycine-treated brucellae. When tested alone, the monocyte agent had no bactericidal or bacteriostatic action on rough or smooth forms of the Rev Is strain. Tests were performed at 37 C by mixing dilutions of extract with 18- to 72-hr cells suspended to final concentrations of 10^8 to 10^9 organisms per ml in the following solutions: 0.08 M Veronal buffer, pH 7.5; 0.066 M phosphate buffer, pH 8.0; the intracellular salts solution of Hirsch, pH 5.6; Tyrode's serum-medium and Albimi brucella broth. The failure to inhibit or kill the cells indicated that the walls were protected from the agent, and in this respect the material behaved like egg-white lysozyme. As with lysozyme and...
GLYCINE-MONOCYTE EXTRACT ACTION ON BRUCELLAE

other gram-negative cells, it was necessary to pretreat the cell to render the substrate available. In the laboratory most of these treatments have been quite drastic e.g., heat, solvent extractions, and freezing and thawing. If the disposal of brucellae within the host involves the action of cellular lysozyme(s), it is probable that in environments in vivo, specific cellular factors modify the wall structure and thereby allow the enzyme(s) to act. As an experimental approach to an understanding of how these biological factors might operate, glycine has been used as their substitute, for it has been found that it causes the Rev Is to become susceptible to the monocyte agent and to egg-white lysozyme.

As indicated in Fig. 2, 3, 4, and 6, simultaneous mixture of glycine with lysozyme or monocyte extract resulted in a rapid onset of lysis and death. The over-all response was dependent upon the physiological state and age of the cell, the relative concentrations of glycine and enzyme, the test medium, and the degree of aeration (Fig. 2). Under the conditions of most tests used in these experiments 0.3 M glycine was required to bring about changes in sufficient numbers of cells to allow detectable measurement of lysis and death.

![Diagram](http://jb.asm.org/)

**Fig. 3.** Comparison of action of monocyte extract and lysozyme on Micrococcus lysodeikticus and glycine-treated Rev Is.

*Brucella* cells, 19-hr growth, harvested in ZoBell’s solution, added to mixture of 4.0 ml Albimi broth, 0.5 ml Tyrode’s or extract in Tyrode’s solution, 0.5 ml 3 M glycine in distilled water, or appropriate amounts of distilled water. Monocyte extracts A to E were prepared from exudates treated in one of three ways described in Materials and Methods. Second series exposed to concentrations of egg-white lysozyme varying from 0.5 to 80 µg/ml, added in 0.5-ml amounts to the reaction mixture. Incubated at 37 C. Initial Klett reading = 50.

*M. lysodeikticus* cells: harvested from 7-day growth on Albimi agar, into distilled water. Exposed to equivalent amounts of the above reagents, at 37 C.
death. This concentration by itself was capable of depressing cell growth, but cellular death was not necessarily a prerequisite for enzyme action. As shown in Fig. 4 and 6, a significant percentage of the glycine-exposed cells remained viable by colony count, but could nevertheless be lysed and destroyed by the addition of monocyte extract or lysozyme.

The action of known amounts of lysozyme and of a series of extracts (A to E) were compared for their relative activities for glycine-treated brucellae and M. lysodeikticus. Those extract preparations with high activity for M. lysodeikticus also exhibited the greatest activity for B. melitensis Rev Is (Fig. 3).

Comparison of rough and smooth strain Rev Is with virulent strain 6015. The vaccine strain Rev Is is capable of multiplying in normal rabbit monocytes in tissue culture media. In the rough state, it is phagocytyzized and rapidly destroyed. The virulent B. melitensis strain 6015 has been shown to multiply rapidly in monocytes. On agar the Rev Is strain develops colonies much more slowly than strain 6015. In other studies, it has been found that glycine in concentrations as low as 0.03 M enables monocytes to depress the growth of smooth Rev Is and accelerate the disappearance of the rough form (Ralston and Elberg, 1960, 1961).

As a supplement to studies of the intramonicytic behavior of these strains, tests were run to compare their relative susceptibility in vitro to the combined action of glycine and lytic agents. Figure 4 shows that cells of rough Rev Is were more easily destroyed than smooth by 0.3 M glycine and 5.0 μg per ml egg-white lysozyme. In both cases a proportion of the initial population survived the treatment. Figure 5 indicates that when the residual cells were centrifuged out of the reaction mixture and placed in fresh 0.3 M glycine and lysozyme they were more resistant than the original population.

As shown in Fig. 6, tests of the combined action of monocyte agent and glycine on the virulent strain 6015 showed it to be more resistant than either the rough or smooth form of Rev Is. Cells of strain 6015 were not killed by 0.3 M glycine alone within 7 hr and the combination of glycine and monocyte agent caused less turbidity decrease than with the Rev Is cells. It appeared therefore in these tests that the ability of the lytic system to destroy the three strains was
inversely related to their relative virulence (rough Rev Is < smooth vaccine Rev Is < virulent 6015). This action could be demonstrated only with relatively young, logarithmic phase cells. Cultures on Albini brucella agar became progressively more resistant as they approached the stationary phase. For example, strain 6015, which multiplied most rapidly, became partially resistant after only 13 to 15 hr at 37 C and the slower growing strain Rev Is after 18 to 22 hr. These observations suggest that the brucella wall undergoes significant changes during growth in those components which tend to protect the cell against this lytic system. The effect of the glycine-lysozyme system on these three forms of brucellae does not necessarily mean that virulence in all brucellae is directly correlated

FIG. 5. Susceptibility of survivors after lysozyme- glycine treatment to fresh additions of lysozyme and glycine. Comparison with initial sensitivity of total population. Test, as for Fig. 4. After 24 hr at 37 C, residual cells centrifuged and resuspended in equivalent amounts of glycine and lysozyme. (The supernatants from the original set of tubes were then tested for residual glycine and lysozyme by adding a suspension of previously unexposed young cells. These underwent lysis in the presence of glycine and lysozyme, indicating the presence of considerable amounts of these two agents.)

FIG. 6. Effect of glycine and monocyte extract on Brucella melitensis strain 6015 and on rough and smooth forms of strain Rev Is. Cells harvested into saline after 18 hr growth on Albini. 0.5 ml added to mixtures containing 4 ml 0.01 m phosphate buffer (pH 7.0), 1 ml Tyrode’s solution, 0.5 ml of 3 m glycine, and 0.5 ml monocyte extract (final dilution 1/25, prepared by freeze-thawing 5.8 X 10^8 monocytes in 20 ml Tyrode’s solution). Tubes incubated at 37 C and read turbidimetrically. Samples for viable count were diluted in ZoBell’s buffer and 0.1 ml aliquots were spread on the surface of brucella agar.
with lysozyme resistance; however the possibility remains a consideration for further study.

**Action of other enzymes on glycine-treated brucellae.** To determine whether glycine causes additional components on the brucella wall, in the underlying membrane, or in its internal constituents, to become susceptible to enzyme action, a series of purified enzyme preparations was added to 18-hr cells in the presence of 0.3 M glycine. Table 2 summarizes the observations made with smooth Rev Is. Of ten enzymes only the lysozymes caused extensive lysis and release of viscous deoxyribonucleic acid. Trypsin effected a slow reduction of turbidity after 14 hr but in this case there was no release of viscous nucleic acid. Together with lysozyme or extract, trypsin exerted an additive effect on the glycine-treated cells. In the absence of glycine, neither trypsin nor lysozyme, alone or in mixture, affected the cells. The results suggest that the effect of glycine

**TABLE 2. Action of enzymes on glycine-treated strain Rev Is in Alibi broth**

<table>
<thead>
<tr>
<th>Cell treatment</th>
<th>% Lysis after 2 hr</th>
<th>% Lysis after 14 hr</th>
<th>Viscosity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control in Alibi broth</td>
<td>0</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>0</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>+ Trypsin</td>
<td>0</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>Extract</td>
<td>0</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>+ Trypsin</td>
<td>0</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>Glycine</td>
<td>10</td>
<td>13</td>
<td>-</td>
</tr>
<tr>
<td>+ Papain</td>
<td>10</td>
<td>13</td>
<td>-</td>
</tr>
<tr>
<td>+ Chymotrypsin</td>
<td>10</td>
<td>13</td>
<td>-</td>
</tr>
<tr>
<td>+ Ribonuclease</td>
<td>10</td>
<td>13</td>
<td>-</td>
</tr>
<tr>
<td>+ Deoxyribonuclease</td>
<td>10</td>
<td>13</td>
<td>-</td>
</tr>
<tr>
<td>+ Hyaluronidase</td>
<td>10</td>
<td>13</td>
<td>-</td>
</tr>
<tr>
<td>+ Lipase</td>
<td>10</td>
<td>13</td>
<td>-</td>
</tr>
<tr>
<td>+ d-Amino oxidase</td>
<td>10</td>
<td>13</td>
<td>-</td>
</tr>
<tr>
<td>+ Cysteine</td>
<td>10</td>
<td>13</td>
<td>-</td>
</tr>
<tr>
<td>+ Extract</td>
<td>23</td>
<td>42</td>
<td>+</td>
</tr>
<tr>
<td>+ Lysozyme</td>
<td>35</td>
<td>70</td>
<td>+</td>
</tr>
<tr>
<td>+ Trypsin</td>
<td>13</td>
<td>24</td>
<td>-</td>
</tr>
<tr>
<td>+ Extract + trypsin</td>
<td>30</td>
<td>50</td>
<td>+</td>
</tr>
<tr>
<td>+ Lysozyme + trypsin</td>
<td>41</td>
<td>79</td>
<td>+</td>
</tr>
</tbody>
</table>

Cells (18 hr) suspended in 3.5 ml Alibi broth + 0.5 ml 3 M glycine and 0.5 ml of enzyme (100 
\( \mu g/ml \)); volume adjusted to 5 ml with distilled water. Incubated static at 37C. Cell input was 66 Klett units. Similar results were obtained with washed suspensions in 0.01 M phosphate buffer, pH 8.0, containing 0.002 M Mg++, Ca++, Mn++ as the chloride salts.

**FIG. 7.** pH activity curve for monocyte extract and lysozyme on Micrococcus lysodeikticus and brucella cells.

is more specific than general and involves structures or mechanisms which normally protect the mucopolysaccharide wall material against lysozyme action.

**Other properties of the monocyte agent: heat stability, trypsin resistance.** The above tests suggested most strongly that the active ingredient in the monocyte extract was a cell lysozyme. In its trypsin resistance (10 \( \mu g \) per ml) and heat stability, it also resembled classical egg-white lysozyme. It withstood boiling for 5 min at pH 4.0 and was only partially inactivated at pH 7.4.

Samples of the monocyte extract and egg-white lysozyme were tested over the range pH 6.4 to 8.0 in a 0.01 M phosphate buffer-20% Tyrode’s solution, as shown in Fig. 7, with glycine-treated brucellae, both lysozyme and extract were more active at pH 8.0 than at 6.4. There was no sharp peak of activity. Tests of glycine alone showed only a small amount of lysis at all pH values, so the effects with the combined agents could be ascribed to their lysozyme moieties. It is possible that the true pH optimum was obscured by a slow secondary response such as the actual disintegration of the bacteria. In contrast to brucellae, egg white lysozyme showed an optimum for *M. lysodeikticus* in the range of pH 7.5, whereas the monocyte
material showed a steady increase of activity between pH 6.4 and 7.8, perhaps reflecting differences between the two agents.

**DISCUSSION**

*Relationship of lytic agent from monocytes to other previously described bactericidal and bacteriolytic agents from white cells.* The agent in the extracts which causes lysis of glycine-treated brucellae does not appear to be related to the phagocyt of Hirsch (1956a, b), or to the leukins characterized by Skarnes and Watson (1956). The former agent exhibited an acid pH optimum and although its activity was predominantly against gram-negative bacteria, it did not cause lysis. The material described as leukin, although showing similarities to the monocyte agent in its pH optimum at 8.2 and heat stability, is nonlytic and trypsin susceptible.

Gengou (1921) described a material from leukocytes, but not from monocytes, which was lytic for gram-negative bacteria, heat stable in acid, and nondialyzable. Lysozyme (Fleming, 1922), and the leukozymes A, B, and C of Amano et al. (1954, 1955, 1956), all cause lysis of various gram-positive and gram-negative bacteria, provided their substrates are available on the surface of the living cell, its untreated wall, or in chemically or metabolically altered walls of normally resistant cells. The material in the monocyte extract appears to contain a lysozyme, since the activities of the preparations for brucellae are correlated with their action on *M. lysodeikticus*. In addition, of a series of purified enzymes, only lysozyme is capable of lysing glycine-treated cells and releasing deoxyribonucleic acid. Other properties which suggest a lysozyme-like nature are its heat stability and trypsin resistance; but further identification awaits chemical purification of the agent and analysis of the components released from brucella walls by its action.

*On the role of tissue lysozyme(s) in infection.* The presence of lysozyme in various tissues and fluids directed attention to the possibility of its function in protecting the body against bacterial invaders (Fleming, 1922). The purified material, however, is inactive against many pathogens, especially gram-negative bacteria, despite the fact that the mucopolysaccharide substrate is widely distributed (Salton, 1953; Cummins and Harris, 1956). Many bacteria are endowed with protective structures or mechanisms which prevent access by lysozyme. These may involve (i) specific groupings on the mucopolysaccharide substrate itself (Brumfitt, Wardlaw, and Park, 1958; Abrams, 1958), which might hinder attachment of the enzyme to reactive sites; (ii) materials which bind and inactivate the enzyme (Salton, 1956b); or (iii) masking materials which might cover the substrate (Weidel, 1958).

In the case of gram-negative bacteria, a number of physical and chemical treatments have been used to render strains susceptible to lysozyme: mild alkali (Nakamura, 1923); solvents (Becker and Hartsell, 1955; Warren, Gray, and Bartell, 1955); freezing and thawing (Kohn and Syzbalski, 1959); chelating agents (Repaske, 1958); and antibiotics (Newton, 1956; Warren, Gray, and Yurchenco, 1957). In addition there appear to be biological agents capable of changing cell resistance. Specific immune serum affects *Escherichia coli*, *Vibrio cholera*, and other bacteria so that they may be lysed by complement and a leucozyme from polymorphonuclear white cells (Amano et al., 1954, 1956; Inai et al., 1954). Factors, possibly wall lysins, in the peritoneal cavity of mice produce transformation of gram-negative pathogens into protoplast-like bodies (Carey, Muschel, and Baron, 1959).

In the present work lysozyme was shown to be inactive against brucellae unless the cells were also exposed to glycine. In other studies, it was found that glycine also affected the yields of brucellae in tissue cultures of rabbit monocytes (Ralston and Elberg, 1960), but under these conditions it was active at lower concentrations. Glycine has been used to induce lysis and abnormal morphology of a number of bacteria including *Brucella abortus* (Gerhardt, 1958). The mode of action is as yet ill defined. It might act by chelating essential metal ions, similar to the findings of Repaske (1958) with Versene. Perhaps it causes an imbalance of wall synthesis, such as has been observed by Shockman, Kolb, and Toennies (1958). Perhaps it has multiple effects in the test tube where it substitutes for a variety of factors in the monocyte. This may be the reason it is required in greater concentration than in experiments with intact monocytes.

These studies, in themselves, in no way prove that lysozyme(s) is involved in the destruction of brucellae in infectious processes. However, the fact that a simple amino acid, such as glycine, can be shown to render the cells susceptible,
suggests that in vivo other metabolites might profoundly alter wall structure and, consequently, resistance to tissue enzyme(s). This might also apply to other monocyte-bacterial systems. In fact, Fong, Chin, and Elberg (1961) have recently demonstrated a series of changes which are impressed upon mycobacteria when they are passed directly through generations of intra-monocytic growth. Losses in mouse lethality, neutral red binding, increased sensitivity to sodium oleate, among others, were determined. These changes were not maintained when growth conditions in vitro were restored. The data supports the view that monocyte factors may induce important changes in the cell surface during intramonicytic growth and that such changes, in their early stages, result in far reaching but reversible alterations in properties relating to the virulence of the strain. Ultimately the demonstration of a role for lysozyme, if any does exist, will depend upon the reconstruction in vitro of a multiplicity of factors, each of which might be important in potentiating stepwise degradations of the bacterial surface.

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