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PRODUCTION AND CHARACTERIZATION OF *BRUCELLA* SPHEROPLASTS

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ABSTRACT

HINES, WILLIAM D. (University of Chicago, Chicago, Ill.), BOB A. FREEMAN, AND GARY R. PEARSON. Production and characterization of *Brucella* spheroplasts. J. Bacteriol. **87**:438-445. 1964.—Spheroplasts of *Brucella* were induced by glycine, penicillin, and a combination of the two. These spheroplasts lacked cell-wall endotoxin but did react with antiserum prepared against cell wall and against whole cells. Phase-contrast photomicrographs and electron micrographs are presented, which show no morphological difference between the protoplasts produced by penicillin and by glycine. The glycine and glycine-penicillin spheroplasts were osmotically sensitive, would not reproduce, and only a small percentage reverted to the bacillary form. Penicillin spheroplasts would not reproduce and were not osmotically sensitive. Penicillin spheroplasts showed significant reversion when induced by low concentrations of penicillin, but the rate of reversion decreased with increasing antibiotic concentration. Penicillin and glycine spheroplasts adsorbed brucellaphage at a reduced rate compared with normal *Brucella*, but spheroplasts produced by the combined action of penicillin and glycine failed to demonstrate brucellaphage adsorption.

Perkins (1963) summarized much of the available information on the nature of the bacterial cell wall, and noted that cell walls of the gram-negative bacteria are much more complex than those of gram-positive bacteria. Kellenberger and Ryter (1958), in an electron microscopic study of thin sections of phage-infected *Escherichia coli*, observed that the cell wall was composed of three layers, each 20 to 30 A thick. Weidel, Frank, and Martin (1960) fractionated cell walls of *E. coli* B and also found three layers. They described an inner hard layer composed of spherical particles held together by mucopeptides made up of alanine, glucosamine, muramic acid, diaminopimelic acid, and glutamic acid; the spheres contained amino acids not associated with the peptide. Also present was a middle, soft lipopolysaccharide layer and an outer, soft protein-lipid layer.

This complexity of structure has been an obstacle in producing true protoplasts of gram-negative bacteria. Many treatments have been devised that cause the gram-negative bacteria to become osmotically sensitive, to lose the capacity to adsorb phage, and to lose much of the diaminopimelic acid and hexosamines normally found in the cell wall. In no case, however, has it been possible to satisfy all criteria for the complete absence of cell wall. This may be difficult to accomplish, since both cell wall and cytoplasmic membrane contain lipoprotein components, and complete dissolution of the wall may also result in damage to the cytoplasmic membrane. In contrast, true protoplasts, i.e., completely lacking in cell walls, have been repeatedly described for gram-positive microorganisms (*see* McQuillen, 1960).

The possibility of producing protoplasts or protoplast-like forms of several strains of *Brucella* was investigated.

MATERIALS AND METHODS

Cultures. The cultures used in this investigation were *B. suis* 32P, *B. abortus* 2308 (CO_2-requiring), *B. abortus* 1279, *B. melitensis* 2452, and a culture of *B. abortus* R19 and its specific brucellaphage described by McDuff, Jones, and Wilson (1962). The cultures were smooth by the oblique-light technique of Henry (1933) and the acriflavine test of Braun and Bonestell (1947). Stock cultures were maintained on 2.0% Tryptose agar slants and stored at 5 C. The cells used for the production of spheroplasts were from 24-hr cultures in Tryptose broth, grown with shaking at 37 C.

Spheroplast production. Brucella cells to be converted to spheroplasts were washed by centrifugation from broth culture. Approximately 10^9 cells were added to a flask containing 30 ml of autoclaved Tryptose broth with 0.2 M sucrose. To induce penicillin spheroplasts, penicillin G was added to a final concentration of 1.5 units per ml. Glycine spheroplasts were produced by the addition of 2.0% (w/v) glycine to the medium.

All preparations were incubated at 37 C with

gentle rotation for 48 hr. The suspensions were examined by phase microscopy at the end of this time, and the extent of spheroplast formation was calculated from Petroff-Hausser counts.

Endotoxin titration. Spheroplasts were titrated for endotoxin activity by use of the rabbit epidermal sensitivity test of Larson et al. (1960). The spheroplast preparations of *B. suis* 32P and a control suspension of a 24-hr culture of normal *B. suis* 32P were fixed in 0.4% formalin for 24 hr. They were washed three times in saline, diluted, and 0.2 ml was injected intradermally into rabbits. An erythema 0.5 cm in diameter after 48 hr was taken as the end point.

Phage adsorption. To test spheroplasts for phage adsorption, we employed *B. abortus* R19 and its specific phage. Spheroplasts were reacted with phage at an infection multiplicity of 14 by use of 10^9 bacterial cells in Albimi broth with 0.2 M sucrose. Samples were removed at intervals, diluted 1:100 in Tryptose broth with 0.2 M sucrose, and centrifuged at 1,900 × *g* for 5 min. The supernatant fluid was saved, and the residue was resuspended in 10 ml of Tryptose-sucrose broth and centrifuged again at 1,900 × *g* for 5 min. These supernatants were pooled and assayed for free phage by use of the soft-agar technique of Adams (1959). The residue was suspended in 1.0 ml of Tryptose-sucrose broth and assayed for infective centers by the soft-agar technique. As a control, *B. melitensis* 2452 was used to assay for nonspecific adsorption, since this strain does not possess receptors for the phage; adsorption was insignificant.

Complement fixation. Antigen titrations were performed on spheroplasts and normal cells by use of a modification of the 50% hemolytic end point complement-fixation test (Taliaferro and Taliaferro, 1950). Spheroplasts were fixed with formalin and counted in the Petroff-Hausser chamber. Samples were reacted with an arbitrary amount (0.5 ml) of specific rabbit antiserum in the presence of four 50% units of complement at 37 C for 1 hr. A hemolytic system, consisting of sheep red blood cells and four 50% units of hemolysin, was added and incubated for 0.5 hr at 37 C. The degree of hemolysis was read against a Veronal buffer blank at 640 mμ on the Beckman model B spectrophotometer. By use of a comparable volume and amount of sheep cells lysed with distilled water, 100% lysis was determined. The per cent hemolysis was plotted against the log of the antigen dilution. The number of antigen units (AH_{50}) was taken as the antilog of the antigen dilution showing 50% hemolysis. The amount of antigen is expressed as the number of AH_{50} units per 10^9 cells.

Electron microscopy. Spheroplasts and normal cells were fixed in 0.4% formalin for 24 hr and washed once in saline; a drop containing about 10^7 bacteria per ml was placed on a carbon-coated copper grid and allowed to dry. They were then chromium shadowed at an angle of 90° and observed in a RCA model EMU-2D electron microscope.

RESULTS

Morphology and strain variability. Table 1 shows the differences in the susceptibility of various *Brucella* strains to the spheroplast-inducing properties of glycine. The four *Brucella* strains were exposed to 2.0% glycine for 48 hr. A differential normal cell-spheroplast count of each strain was then made in a Petroff-Hausser chamber, and the percentage of spherical forms was calculated for each. *B. suis* 32P showed the best conversion to spherical form (99+%), and *B. melitensis* 2452 showed the greatest resistance (10% conversion). Because it gave consistently the best conversion, *B. suis* 32P was used in the remainder of the study, except where otherwise noted.

The morphology of the spheroplasts, as viewed by phase-contrast microscopy, appeared to be similar irrespective of the induction method. They appeared to be greater in volume than the normal rod, were spherical, and were usually biphasic with a dark crescent on one side. They were rarely a uniform gray or a dark sphere smaller in volume than the average spheroplast. Figure 1 illustrates the similar features of the glycine-induced and the penicillin-induced spheroplasts, together with normal cells for comparative purposes. The penicillin-glycine spheroplasts were not different in appearance from those shown.

TABLE 1. *Sensitivity of different Brucella to glycine-induced spheroplast formation*

Strain	Per cent spherical forms
B. suis 32P	99
B. abortus 2308	90
B. abortus 1279	40
B. melitensis 2452	10

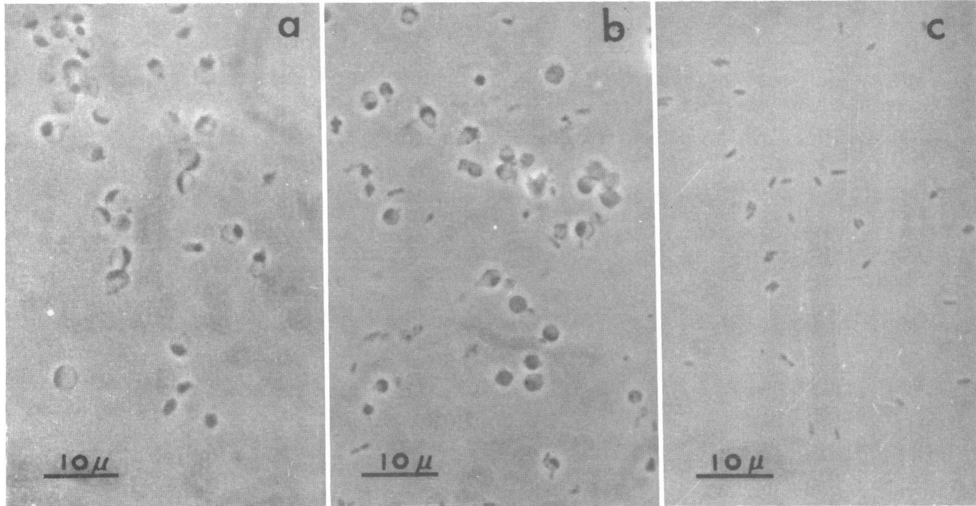

FIG. 1. *Brucella suis 32P, phase contrast (× 1,000). (a) Spheroplasts induced by 2.0% glycine, after 48 hr. (b) Spheroplasts induced by 1.5 units per ml of penicillin, after 48 hr. (c) Normal Brucella.*

To demonstrate more of the fine structure of the spheroplast, electron micrographs were made of whole mounts of normal cells and of the spheroplasts. Only the penicillin and the glycine-induced spheroplasts are shown here, since there is again very little difference in morphology attributable to different inducers. Figure 2a shows an early-stage penicillin spheroplast; Fig. 2b shows a late-stage glycine spheroplast. It is difficult to determine whether the cell in Fig. 2b has lost its cell wall, but the penicillin spheroplast in Fig. 2a has what appears to be an area of residual cell wall near the middle with a bud of protoplasm projecting away from the existing wall. The differences seen here are due to the time of exposure to the inducer.

Penicillin-glycine induction. Since both penicillin and glycine had similar effects on the morphology of *Brucella,* and the effects varied with the strain used, we tested the combined action of penicillin and glycine on *B. suis* 32P. Our data indicate that penicillin and glycine are synergistic in their action on the cell wall. The best spheroplasts are formed with 0.25 units of penicillin per ml and 0.4% glycine. No multiplication was seen under these circumstances, but, if these concentrations were used singly, the cells divided and assumed an aberrant spheroplast-like morphology, i.e., they were not perfectly spherical but were swollen and often misshapen. An increase in the concentration of either or both of these inducers from 0.25 units per ml of penicillin and 0.4% glycine caused less spheroplast formation. If 0.75 units per ml of penicillin and 1.0% glycine were used (concentrations which, if used singly, would induce spheroplast formation), the bacteria were killed and remained as rods. If less than the optimal level of one or both of the inducers was used, division occurred, but the degree of spheroplast formation was decreased.

Spheroplast formation and osmotic sensitivity. The increase in volume and the spherical shape assumed by the normally rod-shaped *Brucella* indicate a change in susceptibility to osmotic pressure. Therefore, it was expected that environments with different degrees of osmotic pressure, as controlled by sucrose concentration, would have different effects on spheroplast formation.

The spheroplasts were induced by 2.0% glycine or by 1.5 units per ml of penicillin in Tryptose-sucrose broth. The penicillin-glycine spheroplasts behaved exactly like the glycine spheroplasts in their susceptibility to osmotic environment. Figure 3 shows the changes in optical density during the formation of the spheroplasts in different concentrations of sucrose.

Morphological changes were followed simultaneously by phase microscopy. At 8 hr, both the penicillin- and glycine-treated bacteria showed abnormal forms. About 50% of the organisms had lost their normal configuration and were swollen to a more oblong shape, or a particular area of the wall was weaker and showed distension. At this time only 2 to 3% of the

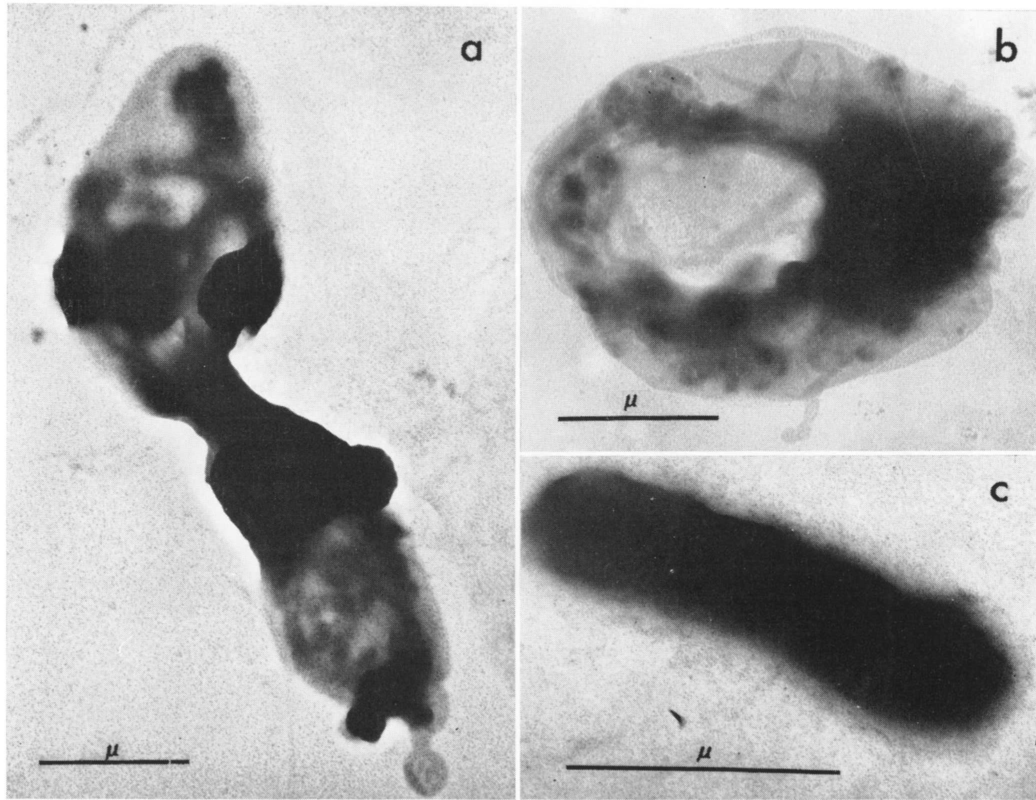

FIG. 2. *Electron micrographs of Brucella suis 32P, whole mounts. (a) Early stage in the formation of spheroplasts induced by 1.5 units per ml of penicillin* (× *20,000*). *(b) Late stage in the formation of spheroplasts induced by 2.0% glycine* (× *21,000*). *(c) Normal Brucella* (× *36,000*).

organisms were spherical; all were spherical at 48 hr in both preparations.

Figure 3 shows that the penicillin-treated *Brucella* steadily increased in optical density irrespective of the sucrose concentration. This increase was at first due to an increase in protoplasmic mass and division of the bacteria. However, as will be discussed below, the rise after a few hours was due to increase in protoplasmic mass only.

The glycine- and penicillin-glycine-treated *Brucella* increased in optical density only in a medium with more than 0.15 M sucrose. At lower concentrations, a steady drop in optical density was seen and observations by phase microscopy showed increasing numbers of lysed spherical forms. However, even at 48 hr, over 50% of the original bacteria were seen as intact spheres.

Reversion and osmotic sensitivity. Another method to detect changes in sensitivity to osmotic pressure is to observe osmotic pressure effects on multiplication of spheroplasts in the absence of inducer. Table 2 shows the effect of sucrose concentrations on spheroplasts. A Petroff-Hausser chamber was used to determine the number of spheroplasts present in the original suspension. Samples were pipetted into tubes with graded concentrations of sucrose. After 2 hr, these suspensions were serially diluted in the corresponding solutions and plated on Tryptose Agar with 0.5 M sucrose. The resulting viable counts are considered to represent reversion of spheroplasts to the rod-shaped form.

All the colonies of both penicillin spheroplasts and glycine spheroplasts were composed of rods. About 25% of the penicillin-induced spheroplasts could revert; sucrose had no effect on this reversion. At best, only 1.4% of the glycine-treated *Brucella* could revert, and the effect of osmotic pressure was similar to that seen by following optical density; similar results were obtained with penicillin-glycine spheroplasts.

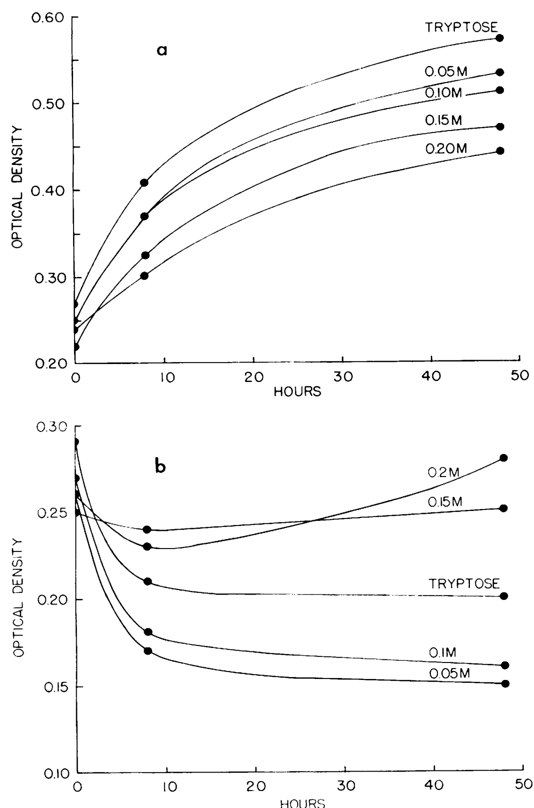

FIG. 3. *Changes in optical density of spheroplast suspensions of Brucella suis 32P in the presence of graded sucrose concentrations in 2.0% Tryptose Broth. (a) Penicillin-induced spheroplasts. (b) Glycine-induced spheroplasts.*

Division of spheroplasts. The limited number of the spheroplasts that are able to revert in the absence of inducer raises a question of the ability of spheroplasts to divide, as spheroplasts, even if kept under proper osmotic conditions. The effect of penicillin on *B. suis* 32P varied with the concentration of penicillin used. At penicillin concentrations of from 0.1 to 1.0 units per ml, the *Brucella* were able to grow and divide as spheroidal forms that tended to stay in chains, but no true spheroplasts were seen. Whether in Tryptose broth or Tryptose broth with 0.2 M sucrose, 1.2 units of penicillin per ml prevented the division of cells, and spheroplasts were formed. At concentrations from 1.2 to 10 units per ml, spheroplasts were formed that could not divide, and above 10 units of penicillin per ml the spheroplasts reverted to a very slight degree only (about 0.01%). If higher concentrations of penicillin were used (20 units per ml or more), the *Brucella* were killed and remained as rods, whether in Tryptose or Tryptose-sucrose broth. Every attempt to show an increase in number of bacteria, in broth medium, at penicillin concentrations greater than 1.5 units per ml was unsuccessful.

Brucella grown in penicillin on Tryptose agar with 0.2 M sucrose were able to divide as spheroplasts. Penicillin, in a range of 0.1 to 4.0 units per ml, induced the *Brucella* to form colonies composed of spheroplasts; lower ranges induced only aberrations in the typical rod shape, but not true spheroplasts. At higher concentrations of penicillin, few colonies were seen.

The effect of varying glycine concentration was similar to that with penicillin. At concentrations of glycine less than 0.5%, the *Brucella* could divide. Their shape was not truly spherical but instead there was a wide variety of aberrant forms present. At concentrations of 1.0 to 4.0%, glycine induced spheroplasts which did not divide and could revert only to a small degree.

Complement fixation. It might be expected that the changes in the ability to multiply and in the structural rigidity of the spheroplast would be reflected in antigenic changes. To test this, rabbit antiserum was prepared against whole *Brucella*,

TABLE 2. *Reversion of Brucella spheroplasts in graded sucrose concentrations*

Sucrose concn	Per cent viable spheroplasts	
	Penicillin-induced	Glycine-induced
M		
0.40	24	1.3
0.30	24	1.3
0.20	25	1.4
0.15	25	1.3
0.10	22	0.20
0.05	22	0.02
Distilled water	22	0.0034

TABLE 3. *Quantitative estimation of antigens of Brucella suis 32P spheroplasts and normal cells as measured by complement fixation*

Antiserum to	Spheroplasts induced by			
	Penicillin	Glycine	Penicillin-glycine	Control
Cell wall	38*	50	29	16
Whole cells	42	32	20	11

* In AH_{50} units. See text for details.

and *Brucella* cell walls. A sample of the whole antiserum was adsorbed with intact whole cells to prepare an antiserum presumably specific to the protoplasm. These antisera were reacted against the several spheroplast preparations, as shown in Table 3. The adsorbed serum is not shown because it failed to fix complement in any of the reactions. The spheroplasts always reacted to a greater extent with both the whole cell and the cell-wall antiserum than did normal cells.

Endotoxin titration. In spite of the failure to demonstrate the loss of any antigens from the spheroplast, we were able to show a complete loss of the *Brucella* endotoxin. Foster and Ribi (1962) demonstrated that this endotoxin was present in isolated *Brucella* cell wall; Table 4 shows that our penicillin and glycine spheroplasts do not contain endotoxin.

Phage adsorption. The bacterial cell wall plays a definite role in the ability of bacteriophage to attach and penetrate the cell. Certain alterations of the bacterial surface might not effect phage adsorption, and other changes might completely block it. As described earlier, phage adsorption was determined both by infective centers and by the decrease in number of phage in the supernatant. The presence of 0.2 M sucrose in the Albimi broth used for adsorption had no measurable effect on the adsorption of the brucellaphage. Figure 4 shows the results of the phage adsorption on normal *Brucella abortus* R19 and on spheroplasts made from this strain. By the infective center method, penicillin spheroplasts and glycine spheroplasts adsorbed only to a slight extent, while penicillin-glycine spheroplasts showed no adsorption. When the number of phage remaining in the supernatant was counted, it was found that the penicillin spheroplasts and the glycine spheroplasts had adsorbed to a much greater extent than was shown by the infective center method, but the

TABLE 4 *Endotoxin of Brucella suis 32P spheroplasts and normal cells as measured by epidermal toxicity in rabbits*

Brucella treatment	Skin-reactive dose*
	μg
Control (normal cells)	0.41
Glycine spheroplasts	43.0
Penicillin spheroplasts	41.0

* See text for details.

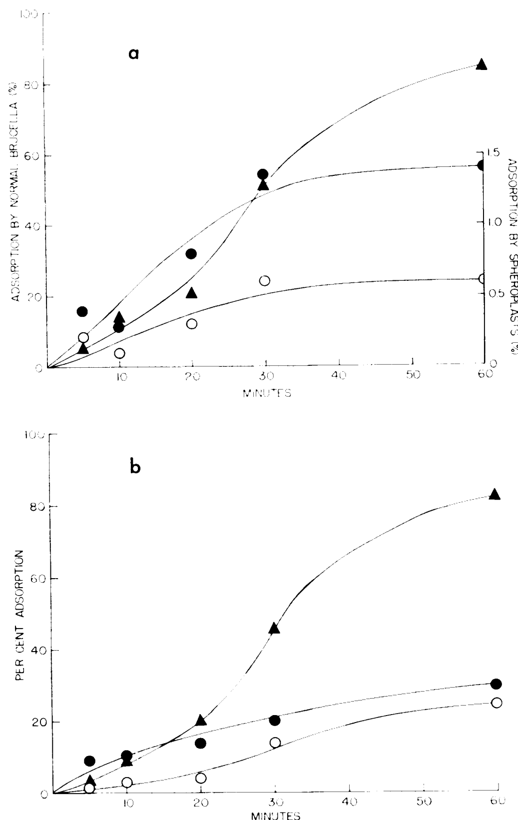

FIG. 4. *Brucellaphage adsorption to Brucella abortus R19 spheroplasts and normal cells. (a) adsorption as measured by infective centers. (b) Adsorption as measured by free phage in the supernatant fluid. Normal Brucella,* ▲; *glycine spheroplasts,* ○; *penicillin spheroplasts,* ●.

penicillin-glycine spheroplasts still showed no phage adsorption.

DISCUSSION

The data presented here show that neither glycine, penicillin, nor combinations of the two are capable of completely eliminating *Brucella* cell wall and thus producing a true protoplast. Further, it is obvious that the different treatments act in different ways, and that glycine and penicillin may have synergistic action on *Brucella* cell walls.

The spheroplasts produced by the combined action of penicillin and glycine most nearly resemble true protoplasts. They are spherical, osmotically sensitive, fail to adsorb specific bacteriophage, fail to revert to bacillary forms, and do not possess endotoxin.

However, insofar as the methods employed were able to detect, a good part of the cellular antigens are still present and no new surface antigens are uncovered, as shown by the failure of antiserum adsorbed with intact *Brucella* to react with the spheroplast. It will be noted, in fact, that the spheroplasts consistently showed greater antigen reactivity than did normal cells. We believe that this is explained by the increased surface area of the spheroplast, which makes antigen more available to antibody. While these findings may indicate that cell wall is still present, the final decision must await proof that there is no sharing of antigens between cell wall and protoplast.

Spheroplasts induced by either glycine or penicillin have some characteristics in common. They are spherical and do not possess endotoxin. Both adsorb brucellaphage, although to a greatly reduced extent when compared with normal *Brucella*, and both still possess antigenic activity. Electron micrographs also demonstrate that the rigidity of the cell wall is lost by treatment with either penicillin or glycine. Although early-stage penicillin spheroplasts are shown to possess residual cell wall (Fig. 2) while glycine spheroplasts do not, experience has shown that this is not attributable to the inducer, but rather is due to the time of exposure. Early stage glycine spheroplasts resemble those in Fig. 2a, and late-stage penicillin spheroplasts resemble those in Fig. 2b. Penicillin and glycine spheroplasts differ, however, in several important aspects. Penicillin spheroplasts are not osmotically sensitive and are capable of reversion to the bacillary form to a significant extent; glycine spheroplasts are sensitive to lowered osmotic pressure and revert only slightly. Park (1958) postulated that both these agents act to inhibit synthesis in a manner that causes an accumulation in the cell of uridine-5′-pyrophosphate derivatives; the precise mechanism is not known.

It is possible that the failure to produce osmotically sensitive spheroplasts with penicillin is due to the use of low concentrations. It is, however, difficult to study higher penicillin concentrations, since there appears to be a lethal effect not associated with spheroplast formation which becomes active at concentrations of 10 units per ml or more. In this case, the penicillin apparently acts very quickly to kill the *Brucella*, leaving the cells covered by a rigid cell wall. At lower concentrations, the synthesis of the cell wall may be only partially inhibited so that the cell can continue to grow until a weakness in the wall develops or until cell-wall synthesis has stopped completely. By this time, the cell could have assumed a spherical configuration.

Still another significant difference between glycine and penicillin spheroplasts is that seen in the phage adsorption studies. Glycine and penicillin spheroplasts both have a decreased ability to adsorb brucellaphage, but glycine-penicillin spheroplasts do not adsorb the phage at all. Since reduced concentrations of penicillin and glycine were used to form the penicillin-glycine spheroplasts it seems unlikely that this is a concentration effect. It may be that the two compounds act synergistically to remove most or all the phage receptors, whereas each one acting separately can remove only a part of the receptor sites.

The difference in the adsorption rates, as measured by the infective-center method and by measuring the number of phage remaining in the supernatant fluid, is probably due to the fragility of the spheroplasts. Lysed spheroplasts, even if they had adsorbed phage, would not support their multiplication and no plaque would be formed.

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LITERATURE CITED

ADAMS, M. H. 1959. Bacteriophages. Interscience Publishers, Inc., New York.

BRAUN, W., AND A. BONESTELL. 1947. Independent variation of characteristics in *Brucella abortus* variants and their detection. Am. J. Vet. Res. **8**:386–390.

FOSTER, J. W., AND E. RIBI. 1962. Immunological role of *Brucella abortus* cell walls. J. Bacteriol. **84**:258-268.

HENRY, B. S. 1933. Dissociation in the genus *Brucella*. J. Infect. Diseases **52**:374-402.

KELLENBERGER, E., AND A. RYTER. 1958. Cell wall and cytoplasmic membrane of *Escherichia coli*. J. Biophys. Biochem. Cytol. **4**: 323-327.

LARSON, C. L., E. RIBI, K. C. MILNER, AND J. E. LIEBERMAN. 1960. A method for titrating endotoxic activity in the skin of rabbits. J. Exptl. Med. **111**:1-20.

McDUFF, C. R., L. M. JONES, AND J. B. WILSON. 1962. Characteristics of brucellaphage. J. Bacteriol. **83**:324-329.

McQUILLEN, K. 1960. Bacterial protoplasts, p. 239-359. *In* I. C. Gunsalus and R. Y. Stanier [ed.], The bacteria, vol. 1. Academic Press, Inc., New York.

PARK, J. T. 1958. Inhibition of cell wall synthesis in *Staphylococcus aureus* by chemicals which cause accumulation of wall precursors. Biochem. J. **70**:2p.

PERKINS, H. R. 1963. Chemical structure and biosynthesis of bacterial cell walls. Bacteriol. Rev. **27**:18-55.

TALIAFERRO, W. H., AND L. G. TALIAFERRO. 1950. The dynamics of hemolysin formation in intact and splenectomized rabbits. J. Infect. Diseases **87**:37-62.

WEIDEL, W., H. FRANK, AND H. H. MARTIN. 1960. The rigid layer of the cell wall of *Escherichia coli* strain B. J. Gen. Microbiol. **22**:158-166.