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Fractionation of Phenol Extracts from *Brucella suis*: Separation of Multiple Biologically Active Components

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Brucella suis endotoxin prepared by a modification of Westphal's method was purified further by gel filtration and ion-exchange chromatography. The latter proved to be the most effective means of separating antigenic components associated with this fraction. All of the toxic activity was located in a single peak from carboxymethyl-Sephadex. Subsequent separation of this material on diethylaminoethyl (DEAE)-Sephadex indicated the presence of two or more toxic components in phenol extracts of *B. suis*. A factor which increases vascular permeability was separated by use of the ion exchanger DEAE-Sephadex, and was associated with the first peak. Its manifestations could be destroyed by heating at 60 C for 30 min or neutralized by reaction with specific antiserum. This permeability factor is believed to be distinct from other toxins which appear in column eluates and which can be assayed by skin induration.

Studies on the factors which influence pathogenicity in *Brucella* have largely centered on the role of endotoxin (2, 16), the various aspects of cellular immunity (5, 9, 17), and the protective immunity rendered by various cell components (1, 12). Because endotoxin exhibits a variety of pharmacological effects, its particular role in virulence has often been suspected (15, 16). However, it now appears that endotoxins derived from smooth *Brucella* strains of differing virulence exhibit similar toxicities when tested in mice (16). It has been shown, too, that heat- or acetone-killed *Brucella* strains of different virulence possess an identical LD_{50} for mice (18). Using more sensitive bioassay techniques, Baker and Wilson (3) concluded that the lethality of endotoxins obtained from *Brucella* strains of high and low virulence does not differ significantly. Therefore, direct correlation between virulence and endotoxin production of *Brucella* is lacking.

It is suggested that indirect mechanisms might be involved. For example, the rate and extent of intracellular multiplication coupled with the relative concentration of endotoxin per infected cell may be important. Pathogenesis may also result from (i) the production of substances which protect the bacteria from host-cell damage (14) or (ii) the production of *Brucella* antigens which may be cytotoxic to host cells. If toxic cellular antigens other than endotoxin are produced, it will be necessary to study their effect in combination with endotoxin to ascertain the role of each in pathogenesis. To test this latter hypothesis, purified preparations of *Brucella* toxins are required.

The acid-insoluble portion of *B. abortus* sonic extract has been shown to be more toxic for immune than for normal macrophages, whereas the acid-soluble part of this preparation is more toxic for normal macrophages (10). The acid-insoluble material contains what is considered to be the classical endotoxin. From this work, it was suggested that more than one cytotoxin is present, and it was further postulated that the acid-soluble toxin may be more exposed in rough *Brucella* or spheroplasts, thus explaining the marked cytopathogenicity observed with these forms.

The purpose of this investigation was to fractionate the toxic components of *B. suis* and to determine the number and kinds of toxins present in phenol-water extracts.

MATERIALS AND METHODS

Cultures. The culture used, *B. suis* 1330, was maintained by growth on 2% Tryptose-agar slants; 18-hr log-phase cultures were harvested in Tryptose broth and stored at −78 C. Cultures for regular use were grown on 2% Tryptose-agar slants and stored at 5 C. In certain experiments, smooth *Vibrio cholerae* 569B and *Escherichia coli* O-55 were employed. They were maintained in the manner of *B. suis*, except that *V. cholerae* was grown in a 1% peptone medium.

Toxin preparation. All endotoxin preparations were made by the Redfearn modification of Westphal's technique as described by Baker and Wilson (3). *Brucella* cultures were grown on large Tryptose-agar slants or in 2% Tryptose broth as suspension cultures

on a rotary shaker at low speed. Cells were harvested and washed three times in 0.85% sodium chloride solution (saline); most preparations contained 5 to 15 g of packed cells which were resuspended in 170 ml of distilled water. Cultures of *V. cholerae* and *E. coli* were handled in the same manner, except that *V. cholerae* was grown in 2% peptone broth at *p*H 8.0.

Cell suspensions were mixed with 190 ml of 90% phenol (w/v), and the mixtures were shaken vigorously for 15 min at 66 C as outlined by Baker and Wilson (3). The resulting water and phenol phases were separated, dialyzed against distilled water, and concentrated to 5 to 10 ml. The phenol-extracted material is termed fraction 5.

Column chromatography. Fraction 5, prepared as described above, was initially separated by column chromatography on Sephadex and Sepharose gels (Pharmacia Fine Chemicals, Piscataway, N.J.).

The gels were equilibrated in either distilled water or 0.1 M sodium phosphate buffer (*p*H 7.2) as recommended by the manufacturer. Cooled and jacketed columns, 2.5 by 50 cm (Pharmacia Fine Chemicals), were packed by pouring a thin slurry of gel into the column which had been partially filled with the eluting fluid. After settling for 24 hr, the gel was washed with a minimum of three bed volumes of the eluting solvent. Flow was maintained by gravity feed through the use of a constant-pressure flask with a head not exceeding 10 to 15 cm of water. The bed volume usually ranged from 150 to 200 ml. The homogeneity of the bed and the void volume of the column were determined by application of Blue Dextran (type 2000, Pharmacia Fine Chemicals). Samples were applied to the top of the column and did not exceed 1% of the total volume.

Effluent was monitored at 254 nm by use of an ultraviolet analyzer (Instrument Specialties Co., Lincoln, Neb.) with a 0.5-cm flow cell and was collected with a fraction collector. Fractions were pooled when required, dialyzed against distilled water at 5 C, and concentrated by low-temperature flash evaporation. The single peak which resulted when fraction 5 was separated on the above gels was further separated by column chromatography on carboxymethyl (CM)- and diethylaminoethyl (DEAE)-Sephadex (Pharmacia Fine Chemicals). The CM-Sephadex was equilibrated with 0.033 M phosphate buffer (*p*H 8.6) and was poured into 2.5 by 50 cm columns. The packed columns, which had final bed volumes of approximately 150 to 200 ml, were washed with several volumes of phosphate buffer. The toxin fraction was applied to the top of the column and was eluted with a continuous linear gradient from 0 to 0.1 M NaCl in 0.033 M phosphate buffer (*p*H 8.6). Ionicity of column eluates was determined by electric conductivity in comparison with NaCl standards.

The DEAE-Sephadex was equilibrated and washed with 0.2 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer (*p*H 8.6). Elution of the sample was accomplished by use of a continuous linear gradient of 0 to 0.1 M sodium chloride in the Tris-hydrochloride buffer. Ionicity was again determined by conductivity. The elution pattern from both types of ion-exchange columns was monitored in the same fashion as the gel filtration columns. Pooled fractions, representing ultraviolet-absorbing peaks, were dialyzed against distilled water, concentrated by flash evaporation, and used in the experiments described below.

Chemical tests. Carbohydrate was determined by either the anthrone test (13) with a glucose standard or by the phenol-sulfuric acid method of Dubois et al. (8). In this test, ribose was employed as the pentose standard, and glucose, as the hexose standard. Since hexose is determined by absorption at 490 nm and pentose by absorption at 480 nm, there may be some cross-reaction at the two wavelengths, thus explaining the somewhat higher values for carbohydrate seen with this method. Dry weights were determined on lyophilized portions of appropriate column fractions.

Serological procedures. Whole *B. suis* 1330 antiserum was prepared by the method of Baughn and Freeman (4). Specific antisera were also prepared with the column fractions, as well as whole fraction 5. Rabbits were immunized with a total of 16 to 20 mg (dry weight) of material per animal; the immunization schedule used was described previously (4).

Agglutinins in these immune sera were titrated by use of Formalin-killed *B. suis* in a standard tube test with a cell density of 3×10^9 cells per ml. Tests were incubated at 56 C for 4 hr, followed by 12 hr at 5 C. Titers are expressed as the highest serum dilution which gave complete agglutination.

To demonstrate the presence of surface antigens, antiserum was absorbed with 5×10^{10} *Brucella* cells. This was accomplished by incubating the antiserum-cell mixture at 37 C for 2 hr with intermittent shaking, followed by incubation at 5 C for 18 hr. After removal of the cells, this serum was titrated for residual agglutinins by the standard tube test.

The technique for immunoelectrophoresis was the same as that described by Baughn and Freeman (4).

Toxicity and toxin neutralization. Fractions were tested for induration toxicity by intradermal injection of 0.1-ml amounts of sample dilution into rabbits and guinea pigs. The reactions were read at 24-hr intervals for 3 days, and maximal reactions were recorded. One unit was that amount of toxin which gave a maximal induration of 0.5 cm (11).

For detection of skin permeability factor (PF) rabbits were inoculated intradermally with 0.1-ml diluted samples, followed 18 hr later by intravenous injection of 0.1 ml of a 1% solution of Niagra Sky Blue dye. Increased vascular permeability at the injection site was indicated by the size of the blue area 6 hr later. One bluing dose was that amount of toxin necessary to give a blue area 0.8 cm in diameter (7).

PF toxin was neutralized by reaction of the different antiserum preparations with the toxin. The mixture was incubated for 30 min at 25 C. Dilutions were then made, and the assay for PF activity was performed as described above.

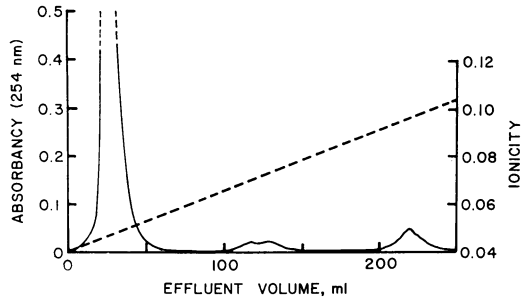

FIG. 1. *Profile of Brucella suis fraction 5 (from Sepharose 2B) eluted from CM-Sephadex with an ionicity gradient in phosphate buffer at pH 8.6.*

RESULTS

Gel filtration of fraction 5. When fraction 5 was separated on Sephadex G-200 and Sepharose 2B and 4B, a single peak of ultraviolet-absorbing material was eluted in the void volume from each of the gels. When this peak was concentrated and immunoelectrophoresis was performed with whole *B. suis* antiserum, four soluble antigens were detected. Two antigens, possibly E and 4 of Baughn and Freeman (4), migrated toward the anode, and one moved slightly toward the cathode. The fourth antigen, which yielded a very diffuse line, did not migrate. These latter two antigens were not found by Baughn and Freeman (4) in whole-cell sonic extracts. This complex material of fraction 5 was of high molecular weight and did not lend itself to further fractionation by gel filtration.

Ion-exchange chromatography of fraction 5. The single peak from Sepharose 2B, containing the detectable antigens, was concentrated and applied to a column of CM-Sephadex. Figure 1 shows the profile of this material eluted with a continuous linear gradient of sodium chloride in 0.033 M phosphate buffer (*p*H 8.6). The majority of the material was not absorbed to CM-Sephadex and was eluted rapidly at low salt concentration as a single peak. All of the dermal toxicity was found in this fraction. Immunoelectrophoresis of this fraction showed that the cathode migrating antigen was weaker and more diffuse, whereas the two anode migrating antigens, E and 4, were much more distinct in a reaction with whole *Brucella* antiserum. This observation suggests that the cathodic antigen was partially removed by CM-Sephadex. No antigens could be demonstrated in the second peak, whereas the last peak contained the nonmigrating antigen; no dermal toxins were detected in either of these latter peaks.

Table 1 shows the protein and carbohydrate content of the various peaks eluted from CM-Sephadex. The first peak contained most of the protein of the sample and the majority of the eluted carbohydrate; all of the dermal toxicity was associated with this peak. The second peak showed small amounts of protein and carbohydrate but no toxicity. The last peak, which contained the nonmigrating antigen, showed traces of protein and contained about 10% of the carbohydrate of the sample.

Figure 2 shows the typical profile when the first peak from CM-Sephadex was applied to DEAE-Sephadex and eluted with a continuous linear sodium chloride gradient (*p*H 8.6). The chemical and physical data on these fractions are given in Table 2. The first fraction appeared near the beginning of the gradient and, by immunoelectrophoresis, contained only the cathodic antigen when developed with whole *B. suis* antiserum.

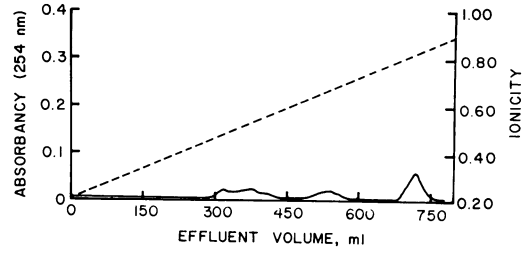

FIG. 2. *Profile of the toxic portion of Brucella suis fraction 5 (from CM-Sephadex) eluted from DEAE-Sephadex with an ionicity gradient in Tris-hydrochloride buffer at pH 8.6.*

TABLE 1. *Chemical and toxic properties of Brucella suis endotoxin fractions eluted from CM-Sephadex*

Fraction	Protein	Carbohydrate			Dry wt	Indurating toxicity
		As hexose	As pentose	Anthrone		
	μg/ml	*μg/ml*	*μg/ml*	*μg/ml*	*μg/ml*	*units/ml*
Whole endotoxin (Redfearn fraction 5)	145	2,620	2,900	485	2,690	160
Column fraction 1	67.5	1,295	1,560	405	1,960	116
Column fraction 2	10	110	110	Trace	100	0
Column fraction 3	Trace	184	150	80	550	0

TABLE 2. *Chemical and toxic properties of Brucella suis toxic fractions eluted from DEAE-Sephadex*

Fraction	Protein	Carbohydrate			Dry wt	Indurating toxicity
		As hexose	As pentose	Anthrone		
	μg/ml	μg/ml	μg/ml	μg/ml	μg/ml	units/ml
Endotoxin (Redfearn fraction 5)	135	2,896	1,630	305	4,330	310
Column fraction 1[a]	12.5	1,320	925	90	1,870	144
Column fraction 2[a]	77.5	810	780	121	1,830	136
Column fraction 3[a]	Trace	610	210	60	510	60

[a] Peaks eluted from DEAE-Sephadex column when applied sample was a toxic column fraction 1 from CM-Sephadex.

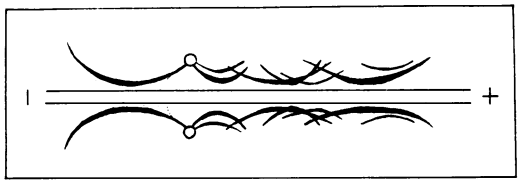

FIG. 3. *Diagrammatic representation of precipitating antigens of Brucella suis fraction 5 extracted for 5 min at 66 C as demonstrated by immunoelectrophoresis.*

This fraction contained only small amounts of protein, but had considerable carbohydrate. The next peak contained the anode migrating antigen, analogous to E of Baughn and Freeman (4), contained most of the protein of the sample, and was rich in carbohydrate. The last peak contained both antigens 4 and E, showed only a trace of protein, and had considerable carbohydrate. Rabbit and guinea pig dermal toxins were present in all three peaks.

Immunological studies. The apparent separation of antigens made possible the preparation of specific antisera to the several purified fractions. By use of these antisera, at least three more anode migrating antigens could be detected. Antiserum to the first fraction from DEAE-Sephadex precipitated only the cathodic antigen, whereas antisera to each of the last two peaks precipitated five anode migrating antigens in a reaction against whole fraction 5. These antigens were, therefore, present in fraction 5, but they did not elicit detectable antibodies when the whole cells were used as immunogens. These several minor antigens are undoubtedly concentrated to immunogenic, but not to precipitable, levels by the phenol extraction.

There was some evidence that the conditions used for phenol extraction destroy some antigens. Extraction time was, therefore, decreased from 15 to 5 min at 66 C. Figure 3 shows the profile obtained when this fraction 5 preparation reacted with homologous antiserum. There appear to be at least seven anodic antigens in addition to the cathodic antigen. As the time of extraction was increased, a progressive loss in number and precipitability of the anodic antigens occurred.

To study the relationship between agglutinogens and precipitinogens, agglutination tests were performed with antisera prepared against the several peaks from CM- and DEAE-Sephadex (Table 3). The antiserum to the first peak from CM-Sephadex agglutinated whole *Brucella* at a dilution of 1:640; that from the second peak, to

TABLE 3. *Comparison of immunogens in column fractions of Brucella suis endotoxin from CM-Sephadex and DEAE-Sephadex*

Ion exchanger	Column fraction	Induced agglutinin titer[a]	Induced precipitins[b]
—	Endotoxin (Redfearn fraction 5)	1:2,560	All antigens
CM-Sephadex	Fraction 1	1:640	Cathode + five anode antigens
	Fraction 2	1:320	None
	Fraction 3	1:10	Nonmigrating antigen
DEAE-Sephadex	Fraction 1[c]	None	Cathode antigen
	Fraction 2[c]	1:80	Five anode antigens
	Fraction 3[c]	1:160	Five anode antigens

[a] Agglutinins induced in rabbits by immunization with column fraction or endotoxin.

[b] Precipitins induced by column fraction or endotoxin as shown by immunoelectrophoresis.

[c] Peaks eluted from DEAE-Sephadex column when applied sample was the toxic column fraction 1 from CM-Sephadex.

TABLE 4. *Comparison of dermal and vascular permeability toxins in Brucella suis endotoxin fractions eluted from DEAE-Sephadex*

Fraction	Induration toxicity[a]	Vascular permeability (BD)[b]
	units/ml	*BD/ml*
Endotoxin (Redfearn fraction 5)	310	80
Column fraction 1[c]	144	70
Column fraction 2[c]	136	0
Column fraction 3[c]	60	0

[a] Induration unit is the amount of toxin which shows 0.5-cm skin reaction within 72 hr.

[b] Bluing dose (BD) is the amount of toxin which gives a bluing diameter of 0.8 cm. See text for details.

[c] Ultraviolet-absorbing peaks eluted from DEAE-Sephadex column when applied sample was a toxic column fraction 1 from CM-Sephadex.

a titer of 1:320. It should be noted that antiserum to the first peak from DEAE-Sephadex contained no detectable agglutinins, whereas material in the second and third peaks induced agglutinins at titers of 1:80 and 1:160, respectively. This indicates that the indurating toxin, found in the first fraction from DEAE-Sephadex, is not associated with an agglutinogen. We believe that toxicity may be associated with the cathodic antigen. In an effort to confirm the observation that toxicity of the fraction is not associated with agglutinogens, the antiserum to this fraction was absorbed with whole cells. Neither the cathode migrating antigen nor toxicity was removed, and thus the cathode antigen is believed to be a subsurface antigen.

Toxicity studies. Other workers have reported toxins in microorganisms which elicit a skin toxicity manifested by increased vascular permeability (6). This is usually termed PF or bluing toxin because it is frequently assayed by increased permeability to a blue dye in the area of the intradermal injection.

Table 4 shows the relative position of the indurating and the PF toxin in the various fractions from DEAE-Sephadex. PF was found in the first peak from DEAE-Sephadex; other fractions from this exchanger had no PF activity. When antiserum to peak one from DEAE-Sephadex reacted with the PF-containing fraction, complete neutralization of this activity occurred. In addition, heating this fraction at 60 C for 30 min destroyed most of the PF toxin. When this heated fraction was subjected to immunoelectrophoresis, the portion of the cathodic antigen nearest the well was missing and assumed to be destroyed.

Brucella and V. cholerae antigens. When a concentrated peptone supernatant from a *V. cholerae* culture was used as an antigen in immunoelectrophoresis and allowed to react with antiserum to the first toxin peak from DEAE-Sephadex, a precipitate line formed at the antigen well. This line was not identical with that formed by the cathodic antigen of *Brucella* demonstrable with this antiserum. The *V. cholerae* PF was immunologically specific; i.e., it was neutralized by homologous antiserum, but not by *Brucella* antiserum.

To determine whether other gram-negative bacteria have a PF which can be extracted in the phenol phase of the Westphal procedure. *E. coli* O-55 and *V. cholerae* 569B Inaba were used. In each case, the water and phenol phases were separated and their PF toxicity was measured. Although a very severe dermal reaction could be elicited with both water and phenol phases of *E. coli*, no PF activity was detected. With *V. cholerae*, a positive bluing reaction occurred with the phenol phase at dilutions as great as 1:64, activity much lower than is found in peptone supernatants.

DISCUSSION

The data from gel filtration studies indicate that *Brucella* fraction 5 is a high molecular weight complex which cannot be readily fractionated by this method. However, CM-Sephadex did afford a method for partial separation of the toxic portions of this fraction. Since immunoelectrophoresis of the first peak from CM-Sephadex indicated that the cathodic antigen had been partially removed, while antigens E and 4 appeared more distinct, it was expected that DEAE-Sephadex would allow separation of the cathodic from the anodic antigens. This proved to be the case, because the first peak from DEAE-Sephadex always contained only the cathodic antigen, and antiserum prepared to this peak precipitated only the cathodic antigen from whole fraction 5. Chemically, this fraction appeared to be mostly carbohydrate, with only a small amount of protein.

The next two peaks from DEAE-Sephadex exhibited, upon immunoelectrophoresis, the anode migrating antigens. Peak two contained most of the eluted protein and a considerable amount of carbohydrate. The last peak appeared to be mostly carbohydrate. Antiserum against either of these peaks precipitated five anode antigens from fraction 5. The fact that all three of the peaks from DEAE-Sephadex showed rabbit and guinea pig dermal toxicity suggests that multiple dermal toxins are present in *B. suis*.

Antiserum to peak one from DEAE-Sephadex contained no detectable agglutinins and absorption with whole cells did not remove antibody to the cathode antigen, indicating that the antigen is

not present on the cell surface. This antigen is probably associated with the dermal toxicity of this column fraction, thus providing a clear demonstration that dermal toxicity in *Brucella* is not always directly associated with agglutinogens. It was also found that a PF toxin is associated only with this column fraction. This observation, coupled with the fact that dermal toxicity is found also in column fractions 2 and 3, strongly suggests that more than one dermal-type toxin is present in *Brucella*. The neutralization and heat denaturation experiments indicate that this PF toxicity is indeed specific. The partial loss of the cathodic antigen upon heating suggests that this part of the antigen is associated with PF activity.

A comparison of phenol extracts of *E. coli* and *V. cholerae* indicates that only *V. cholerae* possesses a PF toxin, thus confirming earlier observations (6). The possible relationship between the PF factors of *Brucella* and *Vibrio* remains unclear.

The discovery of multiple toxic materials in the *Brucella* cell necessitates the evaluation of each component in virulence and pathogenicity of this microorganism. It is particularly important to determine the presence of the PF factor in strains of different virulence. We further believe that the toxic role in *Brucella* cannot be assigned solely to endotoxin.

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LITERATURE CITED

1. Badakhsh, F. F., and J. W. Foster. 1966. Detoxification and immunogenic properties of endotoxin-containing precipitate of *Brucella abortus*. J. Bacteriol. **91**:494–498.
2. Baker, P. J., and J. B. Wilson. 1965. Chemical composition and biological properties of the endotoxin of *Brucella abortus*. J. Bacteriol. **90**:895–902.
3. Baker, P. J., and J. B. Wilson. 1965. Hypoferremia in mice and its application to the bioassay of endotoxin. J. Bacteriol. **90**:903–910.
4. Baughn, R. E., and B. A. Freeman. 1966. Antigenic structure of *Brucella suis* spheroplasts. J. Bacteriol. **92**:1298–1303.
5. Braun, W., A. Pomales-Lebron, and W. R. Stinebring. 1958. Interactions between mononuclear phagocytes and *Brucella abortus* strains of different virulence. Proc. Soc. Exp. Biol. Med. **97**:393–397.
6. Craig, J. 1965. A permeability factor toxin found in cholera stools and culture filtrates and its neutralization by convalescent cholera sera. Nature (London) **207**:614–616.
7. Craig, J. P. 1966. Preparation of the vascular permeability factor of *Vibrio cholerae*. J. Bacteriol. **92**:793–795.
8. Dubois, M., K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith. 1956. Colorimetric method for determination of sugars and related substances. Anal. Chem. **28**:350–356.
9. Elberg, S. S. 1960. Cellular immunity. Bacteriol. Rev. **24**: 67–95.
10. Hinsdill, R. D., and D. T. Berman. 1968. Antigens of *Brucella abortus*. II. Toxicity for macrophages in culture. J. Infec. Dis. **118**:307–316.
11. Larson, C. L., E. Ribi, K. C. Milner, and J. E. Lieberman. 1960. A method for titrating endotoxin activity in the skin of rabbits. J. Exp. Med. **111**:1–20.
12. Markenson, J., D. Sulitzeanu, and A. L. Olitzki. 1962. Immunogenic activity of *Brucella* cell wall. Brit. J. Exp. Pathol. **43**:67–76.
13. Scott, T. A., and E. H. Melvin. 1953. Determination of toxigenic L-phase variants from *Clostridium tetani*. Acta Pathol. Microbiol. Scand. **46**:333–338.
14. Smith, H., and R. B. Fitzgeorge. 1964. The chemical basis of the virulence of *Brucella abortus*. V. The basis of intracellular survival and growth in bovine phagocytes. Brit. J. Exp. Pathol. **45**:174–186.
15. Spink, W. 1956. The nature of brucellosis, p. 216. Univ. of Minnesota Press, Minneapolis.
16. Spink, W., and D. Anderson. 1954. Experimental studies on the significance of endotoxin in the pathogenesis of brucellosis. J. Clin. Invest. **33**:540–548.
17. Stinebring, W. R. 1962. Characteristics of intracellularly grown *Brucella abortus*. J. Infec. Dis. **111**:17–24.
18. Wilson, J. B., S. Kolbye, and P. Baker. 1964. Role of immunity in sensitivity of mice to *Brucella* endotoxin, p. 251. *In* M. Landy and W. Braun (ed.), Bacterial endotoxins, Rutgers Univ. Press, New Brunswick, N.J.