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Antigens of *Brucella abortus*

I. Chemical and Immunoelectrophoretic Characterization

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ABSTRACT

Extracts of *Brucella abortus* 2308S, prepared either by aqueous extraction of sonically ruptured cells or by phenol-water extraction of whole cells, were subjected to various fractionation procedures and then analyzed to determine their immunoelectrophoretic patterns and chemical properties. Fraction A, prepared from sonic extracts, contained at least nine precipitable components when analyzed by immunoelectrophoresis. Of these, five components gave reactions of nonidentity with each other and, hence, represented separate antigens having unrelated determinant groups. Antigenic component IX, found in both the phenol and sonic extracts, did not form a precipitin line in the presence of serum that had been adsorbed with whole cells and was therefore tentatively identified as a "surface" antigen. From several lines of evidence, component IX was thought to be a lipopolysaccharide similar to the AP substance described by Miles and Pirie and shown by them to carry the "abortus" and "melitensis" determinant groups.

There is a striking lack of precise knowledge concerning the identity and biological significance of the antigens of the brucellae. The present investigation was undertaken in an effort to provide information about the number and types of antigens present in *Brucella abortus* and, if possible, to bring about their separation by chemical or physical means so as to allow eventually for a study of their individual biological properties.

The starting material was obtained either by water extraction of cells ruptured by sonic oscillation or by the Westphal phenol-water extraction procedure. Only the fractionation procedures employed and the chemical and immunoelectrophoretic characterization of the various antigens isolated will be dealt with in this communication. The biological significance of these antigens, as determined by their toxicity for monocytes in culture and ability to evoke skin reactions in guinea pigs, will be reported separately.

MATERIALS AND METHODS

Source of microorganisms. All antigenic fractions were prepared from cultures of *B. abortus* 2308S. This strain is a subculture of the one originally obtained from the Agricultural Research Service, U.S. Department of Agriculture. Lyophilized stock cultures of the organism in skim milk (18) were prepared and used as needed to avoid problems arising from population changes. Evidence of smoothness was obtained by the acriflavine test (4) and the crystal violet test (19).

Methods of culture. Subcultures were grown on brucella agar (Albimi) slants. After incubation for 12 hr at 37 C, the growth from each tube was suspended in sterile saline and was used as the inoculum for each flask containing 500 ml of brucella broth (Albimi). The flasks were placed on a rotary shaker at 37 C for 24 hr, after which phenol was added to the flasks to give a final concentration of 0.5% (w/v). The flasks were returned to the shaker for an additional 12 hr at 37 C to insure killing. The cells were sedimented by centrifugation, suspended in saline, and again collected by centrifugation. The washing procedure was repeated three more times by use of distilled water. A sufficient quantity of distilled water was added to 50 g of cells (wet weight) to give a final volume of 60 ml prior to making the sonic extracts. Cells to be used for the phenol extraction procedure were harvested in the same manner, suspended in a minimal quantity of distilled water, and lyophilized.

Sera for immunological studies. The immune serum used for the immunoelectrophoretic studies came from a single pooled harvest, obtained from six cows which had been artificially infected with *B. abortus* 2308S. The agglutination titer of the serum was 1:10,240 when tested by the tube method, with U.S. Department of Agriculture *B. abortus* standard tube test antigen. Phenol was used as the preservative in a final concentration of 0.5% (w/v). Absorbed serum was prepared by mixing 0.4 g of dry, lyophilized *B. abortus* 2308S with 25 ml of immune serum, incu-

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bating in a water bath at 37 C for 30 min, and collecting the supernatant fluid after centrifugation. The procedure was repeated three more times, the dry bacteria being mixed with the supernatant fluid of the previous tube in each case. The final absorbed serum had an agglutination titer of less than 1:20 when tested by the tube method with the standard tube test antigen.

Sonic extracts. Cell breakage was achieved in 1 hr at 3 C by use of a 10-kc, 250-w sonic oscillator. Immediately after this procedure, the suspension was centrifuged at 18,400 × *g* for 30 min at 4 C. The supernatant fluid was set aside, and the cellular debris was resuspended in 60 ml of distilled water and centrifuged in the same manner. The two supernatant fluids were combined and filtered through Whatman no. 1 filter paper. The resulting filtrate was lyophilized and labeled fraction A.

Phenol-water extracts. These were prepared by the Westphal procedure as modified by Redfearn (Ph.D. Thesis, Univ. Wisconsin, Madison, 1960), and were subsequently described in a paper by Baker and Wilson (1). The material recovered from the phenol phase was designated as fraction 5.

Zone electrophoresis in starch. The starch block was made of powdered potato starch (technical grade) and tris(hydroxymethyl)aminomethane-acetic acid buffer adjusted to *p*H 8.2 (ionic strength, 0.02). The starch trough was 35 cm long with a radius of 2.75 cm. Continuous-flow buffer chambers similar to those described by Kunkel and Slater (7) were used. The technique employed was essentially that described by Bodman (3).

Immunoelectrophoresis. The immunoelectrophoretic studies were carried out in a buffered gel consisting of 1.5% agar (Difco) and sodium diethylbarbiturate-hydrochloric acid buffer (*p*H 8.2; ionic strength, 0.385). The gel was supported on 4 by 5 inch (10.2 by 12.7 cm) photographic plates, and the antigens were incorporated into melted buffered agar before being deposited in the antigen wells.

Chemical determinations. Nitrogen was determined by micro-Kjeldahl analysis (6), and the percentage of protein was calculated by multiplying the percentage of nitrogen by a factor of 6.25. The protein content of eluates obtained from starch segments after zone electrophoresis was determined by the Folin-Ciocalteu tyrosine method as modified by Lowry et al. (9). Crystalline bovine serum albumin was used to prepare a standard curve. Carbohydrate was estimated by the method described by Loewus (8), with glucose as a standard. The qualitative test for the presence of polysaccharide in immunoelectrophoretic plates was performed as described by Uriel and Grabar (17), except that the initial oxidation with periodic acid was lengthened to 3 hr.

RESULTS

Nine precipitin lines were observed when fraction A was subjected to immunoelectrophoresis (Fig. 1). If a shorter period of electrophoresis was used, lines I and II crossed lines III, IV, and V. Antigenic component IX was immobilized at the origin and formed a diffuse, heavy precipitin line just inside the antigen well. When fraction A was subjected to immunoelectrophoresis with absorbed serum, a precipitin line was not observed inside the antigen well, suggesting that component IX was of a "surface" nature (Fig. 2). All the other lines were formed in the presence of absorbed serum, although some of them were barely visible. In situations where the precipitin lines crossed each other, the determinant groups were clearly not identical, as was the case for antigenic components III, IV, and V or for components IV, V, and VII. The antigenic relationship among some of the other components, such as V, VI, and IX, was not clear since the lines did not cross under any of the experimental conditions employed.

These results suggested that some of the antigenic components of *Brucella* could be separated on the basis of electrophoretic mobility. A 150-mg amount of fraction A was subjected to zone electrophoresis in starch. The starch block was then cut into 1-cm segments, and the eluants obtained from these were tested by immunodiffusion and were also analyzed for protein (Fig. 3). Besides the difficulty experienced in trying to correlate the protein content of the eluants with the appearance of precipitin lines, it was found that the bulk of the protein remained in the vicinity of the origin. The failure of most of the material to migrate, in addition to the poor recoveries obtained, indicated that the antigens were being strongly bound by the starch. This approach was therefore abandoned, and other methods of separation were sought.

Ammonium sulfate precipitation appeared to be of limited usefulness, since the precipitated antigens were extremely difficult to redissolve. Acid precipitation proved to be of some value, however. A solution containing 60 mg/ml of fraction A was lowered to *p*H 3.5 by the slow addition of glacial acetic acid. The resulting flocculent precipitate was sedimented by centrifugation at 8,000 × *g*. The supernatant fluid, designated fraction B, was filtered through a Seitz filter and was dialyzed until free from acid and yellow color.

The acid-insoluble material was suspended in water, and 1.0 N NaOH was slowly added until *p*H 11.0 was reached. At this *p*H, the precipitate readily went into solution. Glacial acetic acid was then slowly added and the acid precipitation procedure was carried out as before, the process being repeated two more times. The supernatant fluids were discarded in each case, and the final precipitate was suspended in water. The suspension was adjusted to *p*H 7.4 with 1.0 N NaOH and was dialyzed against distilled water. After cen-

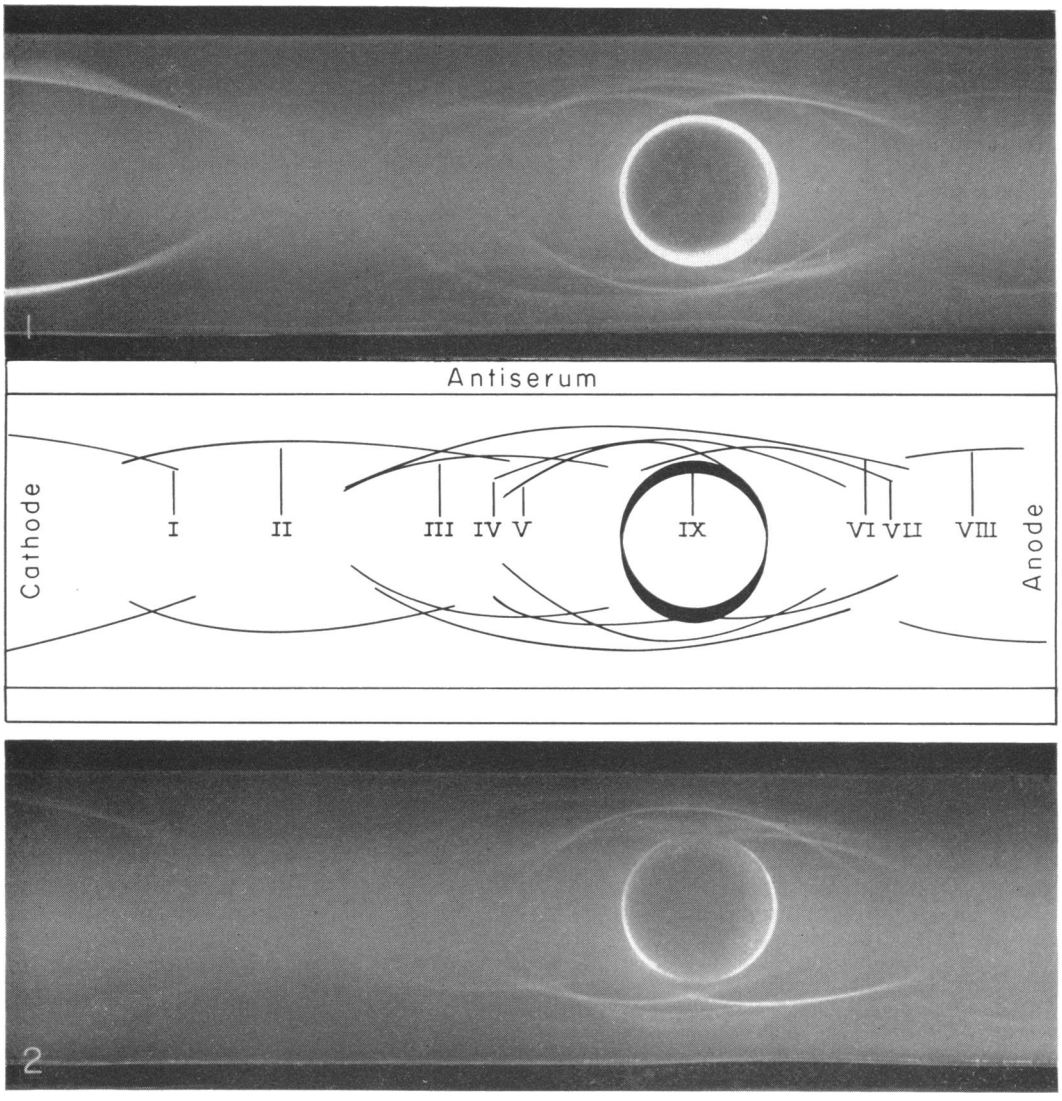

FIG. 1 and 2. *Immunoelectrophoretic analysis of fraction A obtained from extracts of sonically disrupted cells of Brucella abortus. (1) Photograph and drawing showing the location of precipitin lines obtained with unabsorbed serum. Precipitin lines II and III were barely visible in the original photograph. (2) Results obtained with serum absorbed with B. abortus cells. Absence of precipitin line IX revealed the "surface" nature of the immobile antigenic component.*

trifugation at 18,500 × *g*, the slightly opalescent supernatant fluid was collected and designated fraction C.

Immunoelectrophoretic analyses of fractions B and C are shown in Fig. 4 and 5, respectively. Fraction B contained only antigenic components I and II in detectable amounts. Fraction C appeared to be more complex, containing antigenic components with mobilities similar to II, V, VI, VII, and IX found in fraction A. Antigenic component IX was not detectable in fraction C with absorbed serum. Dry-weight analyses indicated that 14% of fraction A was accounted for as fraction B, and that fraction C accounted for 76%.

In a further attempt to isolate the different antigenic components of *B. abortus*, either individually or in small groups, phenol-water extracts were prepared. Immunoelectrophoretic analysis of fraction 5, derived from the phenol phase, revealed four precipitin lines with mobilities similar to antigenic components I, II, VII,

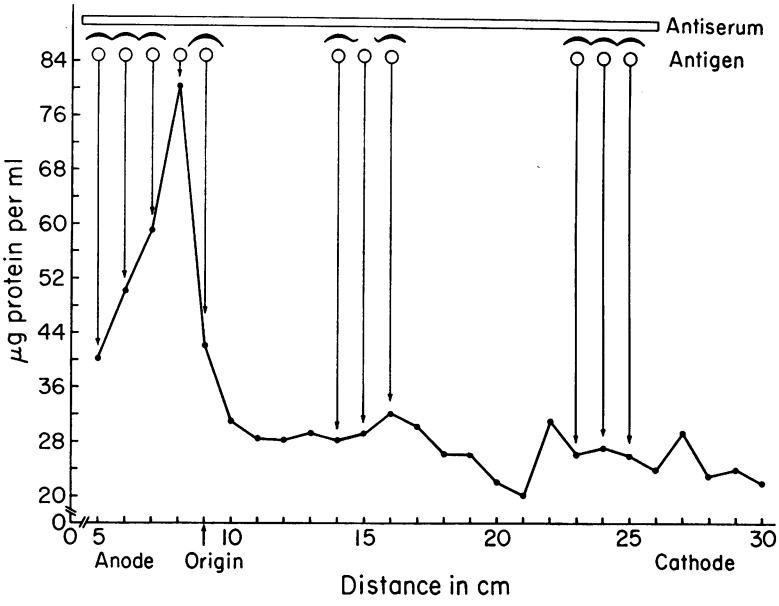

FIG. 3. *Analysis of the eluants obtained after zone electrophoresis of fraction A derived from Brucella abortus. The eluant from each 1-cm starch segment was tested for protein content and examined by immunodiffusion.*

and IX found in fraction A from the sonic extracts. When this immunoelectrophoretogram was stained to show the presence of polysaccharides, only antigenic component IX formed the colored complex indicative of polysaccharides.

By use of the procedure described by Redfearn (Ph.D. Thesis, 1960), fraction 5 was subjected to further fractionation. One volume of cold methanol-sodium acetate reagent was added to fraction 5, and the mixture was held at 4 C for 12 hr. (The methanol-sodium acetate reagent was made by adding 1 part of methyl alcohol saturated with sodium acetate to 99 parts of anhydrous methyl alcohol.) After centrifugation of the mixture at 8,000 × *g* for 15 min, the supernatant fluid was discarded and the precipitate was dissolved in distilled water. This precipitation procedure was repeated two more times, and the final precipitate was dissolved in its initial volume of distilled water. Four volumes of glacial acetic acid were then added slowly with stirring, and the solution was allowed to stand at room temperature for 1 hr. The precipitate was collected by centrifugation at 8,000 × *g* for 15 min and was dissolved in its initial volume of distilled water. After two additional acid precipitations under the same conditions, the final precipitate was dissolved and dialyzed against distilled water until free from acid. The solution was then centrifuged at 8,000 × *g* for 30 min, and the supernatant fluid was designated as fraction 5a. Immunoelectrophoretic analysis of fraction 5a revealed only a single precipitin line within the antigen well, indicating that this fraction contained only antigenic component IX in detectable amounts (Fig. 6). When fraction 5a was first heated to 100 C in the presence of 1% acetic acid for 1 hr, dialyzed free from acid, and then subjected to immunoelectrophoresis, a second precipitin line was observed just outside the antigen well (Fig. 7).

The protein and carbohydrate contents of the various antigenic fractions are listed in Table 1. A qualitative test for lipid (5) showed the latter to be present in fractions A, C, 5, and 5a.

DISCUSSION

Fraction A prepared from the sonic extracts of *B. abortus* 2308S contained at least nine precipitable components when studied by immunoelectrophoretic analysis. In those instances where the components produced precipitin lines which crossed each other, there can be no question about the nonidentity of the determinant groups. On this basis, *B. abortus* contains a minimum of five distinct antigens, represented by antigenic components I, II, III, IV, and V of fraction A. This agrees with the number of antigens enumerated by other investigators (2, 15) working with *B. abortus*.

Although many of the precipitin lines formed in the presence of absorbed serum were extremely faint, as compared with those formed with un-

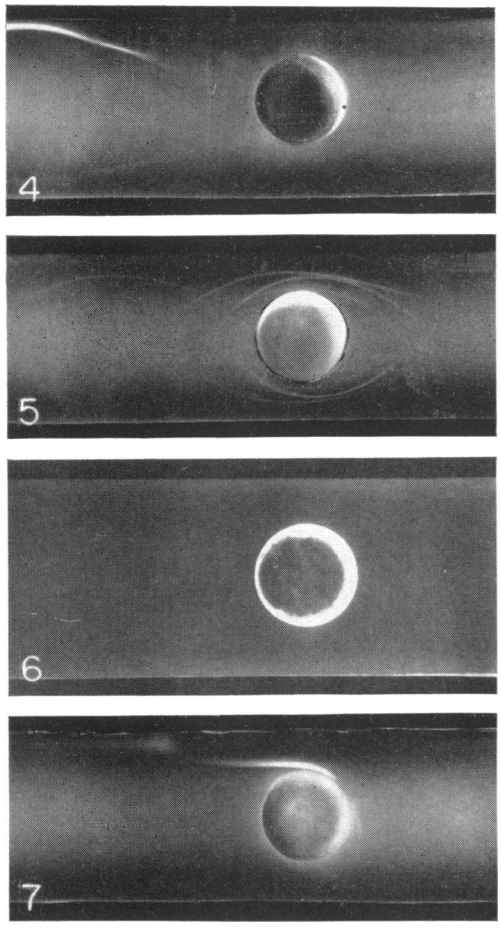

FIG. 4–7. *Immunoelectrophoretic analysis of various antigenic fractions obtained from Brucella abortus. The upper serum troughs were filled with unabsorbed serum, and the lower troughs, with serum absorbed with B. abortus cells, except for Fig. 6 where only unabsorbed serum was used. (4) Fraction B, the acid-soluble portion of fraction A obtained from extracts of sonically disrupted cells. (5) Fraction C, the acid-insoluble portion of fraction A. (6) Fraction 5a, isolated by methanol and acid precipitation of fraction 5 obtained from phenol-water extracts. (7) Fraction 5a after partial acid hydrolysis.*

absorbed serum, at least eight lines were still detectable with fraction A. Only component IX could be tentatively identified as a "surface" antigen, since it formed no precipitin line with the absorbed serum.

Separation of fraction A by acid precipitation yielded two fractions, B and C, which possessed quite different chemical and antigenic characteristics. Fraction B is made up largely of carbohydrate, contains no lipid, and is composed of the fast-moving antigenic components I and II.

TABLE 1. *Chemical analyses of the various antigenic fractions obtained from Brucella abortus 2308S*

Fraction	Nitrogen	Protein	Carbohydrate
	%	%	%
A	15.0	93.8	3.9
B	1.6	10.2	84.6
C	8.1	50.7	20.1
5	7.1	44.4	16.2
5a	5.8	36.2	10.9

Hence, this preparation was of special interest in the toxicity tests which followed this study, and which showed that fraction B was more toxic for normal monocytes than for monocytes obtained from guinea pigs vaccinated with *B. abortus* strain 19.

Immunoelectrophoretic analysis of fraction 5, obtained from the phenol phase of phenol-water extracts, revealed antigenic components with mobilities similar to components I, II, VII, and IX of fraction A. Further purification of this fraction with methanol and acetic acid produced a substance (fraction 5a) which upon immunoelectrophoretic analysis was shown to contain only antigenic component IX in detectable amounts. Mild acid hydrolysis of fraction 5a released a diffusible, but electrophoretically immobile, antigenic component which formed a precipitin line just outside the antigen well after immunoelectrophoresis. The appearance of this line closely resembled that of the precipitin line formed by the major antigen present in the 0.5% phenol extracts of *B. melitensis* prepared by Schneider (Ph.D. Thesis, Univ. Wisconsin, Madison, 1961). The antigen was thought by Schneider to be identical to the component isolated from the phenol extracts of *B. melitensis* by Miles and Pirie (10–12) and described as a formyl derivative of an amino polyhydroxy sugar (AP substance).

In addition, Redfearn (Ph.D Thesis, 1960) has analyzed acid-hydrolyzed fraction 5a derived from both *B. abortus* and *B. suis* by immunodiffusion. Each of these fractions contained two antigenic components, one of which had reactions of identity, whereas the other showed reactions of partial identity with the antigens present in unhydrolyzed fraction 5a obtained from *B. melitensis*. Thus, on the basis of its immunoelectrophoretic behavior and cross-reactions in double diffusion, antigenic component IX, contained in fraction 5a derived from *B. abortus*, appears to be related to the major antigen of *B. melitensis*.

Although much remains to be resolved, these results added to the findings of Wilson and Miles (20), Miles and Pirie (10–14), and Paterson et al.

(16) support the concept that all three species of *Brucella* contain an AP-like substance which carries the A and M antigenic determinants. Furthermore, this AP-like substance seems to be closely associated with a cell wall lipopolysaccharide (antigenic component IX). There are also a number of separate antigens (antigenic components I, II, III, IV, and V) which probably are not principally associated with the cell surface and which do not appear to be species-specific. The present study, though not achieving complete separation of these antigenic components, did provide a number of fractions that could be used in a subsequent study of their biological activities.

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