

Differences Between *Brucella* Antigens Involved in Indirect Hemagglutination Tests with Normal and Tanned Red Blood Cells

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Brucella antigens capable of sensitizing normal and tanned sheep red blood cells for indirect hemagglutination were compared with antigens involved in agglutination, gel diffusion, and immunoelectrophoresis. Hyperimmune rabbit sera, before and after absorption with various antigenic preparations from smooth and rough *B. abortus*, were used in the tests. Normal erythrocytes could be sensitized with a NaOH-treated ether-water extract (EW-T) of smooth *Brucella*. Tanned erythrocytes could be sensitized with a water-soluble extract from ultrasonically disrupted smooth or rough *Brucella*. The EW-T produced a single precipitation band and the water-soluble antigens produce 6 to 23 bands in immunoelectrophoresis with unabsorbed sera. After absorption of antisera with water-soluble extracts from smooth or rough *Brucella* cells or from smooth or rough cell walls, the hemagglutinins for sensitized tanned erythrocytes and the precipitins for water-soluble antigens were removed. Absorption with living smooth or rough *Brucella* cells or with EW-T did not remove these antibodies. The precipitins and hemagglutinins for the antigen EW-T, and agglutinins for smooth cells, were absorbed by smooth antigens but not by rough antigens. It appears that the antigens which sensitize tanned erythrocytes and diffuse through agar gels are present in both smooth and rough forms and may be situated in the cytoplasm or in the internal part of the cell wall, whereas the agglutigen and the antigen which attaches to normal erythrocytes are surface antigens found only on the smooth *Brucella* cell.

Applications of the indirect-hemagglutination test in brucellosis, with the use of sensitized normal erythrocytes, have been reported by Carrère and Roux (6), Barber et al. (2), Khristoforov (13), and Becht (4). Ris and Te Punga (18) sensitized tanned erythrocytes with soluble antigens prepared from a rough species, *Brucella ovis*. R. Diaz and A. Chordi (Bacteriol. Proc., p. 46, 1966) showed that a water-soluble extract from smooth *B. melitensis* was able to sensitize tanned erythrocytes. Rabbits immunized with acetone-killed *B. melitensis*, in either the smooth or rough phase, produced indirect hemagglutinins of the same titer, and there was no difference in titer whether the tanned cells were sensitized with antigen from smooth or rough *Brucella*. In further studies, Diaz, Jones, and Wilson (8) showed the antigenic relationship between smooth and rough *B. melitensis* and *B. ovis* by the indirect-hemagglutination test and by immunoelectrophoretic analysis with the use of water-soluble antigens.

We were unable to sensitize normal erythro-

cytes with the water-soluble antigens, whereas NaOH-treated ether-water extract of *Brucella* did sensitize normal erythrocytes. This study was carried out to determine the differences between the activities of the two antigenic preparations (i.e., the water-soluble antigens which sensitized tanned red cells and the treated ether-water extract which sensitized normal red cells), by correlation with other immunological methods such as agglutination, agar-gel diffusion, and immunoelectrophoresis.

MATERIALS AND METHODS

A list of the preparations employed in this study is given in Table 1.

Antigenic preparations. Strains of *B. abortus* employed in this study have been described previously (1). Strain 2308, a virulent strain, and strain 11, an avirulent strain, both of smooth colonial morphology, and strain 11R, a rough mutant of strain 11, were grown on Trypticase Soy Agar or in Trypticase Soy Broth (BBL) on a shaker for 2 to 3 days at 37 C.

Harvested organisms were suspended in distilled water, and 3 volumes of chilled acetone were added to

TABLE 1. *Antigens and antisera used*

Abbreviation	Description
<i>Antigens</i>	
SA-S	Water-soluble extract from acetone-killed smooth <i>Brucella abortus</i> strain 2308 or 11 which had been ultrasonically treated
SA-R	Water-soluble extract from acetone-killed rough <i>B. abortus</i> strain 11R which had been ultrasonically treated
SA-SL	Water-soluble extract from living <i>B. abortus</i> strain 11 which had been ultrasonically treated
CW-S	Crude cell wall preparation from acetone-killed smooth <i>B. abortus</i> strain 2308 or 11 which had been ultrasonically treated
CW-R	Crude cell wall preparation from acetone-killed rough <i>B. abortus</i> strain 11R which had been ultrasonically treated
EW	Ether-water extract from smooth <i>B. abortus</i> strain 2308
EW-T	EW treated with NaOH
S	Living smooth <i>B. abortus</i> strain 11
R	Living rough <i>B. abortus</i> strain 11R
<i>Antisera</i>	
Anti-SA-S	Rabbits hyperimmunized with SA-S in adjuvant
Anti-CW-S	Rabbits hyperimmunized with CW-S in adjuvant

1 volume of cell suspension. They were held overnight at -20°C , and the cells were then washed three times in cold acetone and dried in a desiccator over calcium chloride. The cells were resuspended in distilled water and treated in a Raytheon sonic oscillator for 2 hr. Additional distilled water was added to ensure thorough suspension. The material was dialyzed against distilled water at 4°C for 2 days and then was centrifuged at $5,500 \times g$ for 1 hr.

The supernatant fluid was concentrated by pervaporation (Rotavapor 'R,' Büchi, Switzerland) and stored at -20°C for 3 days. Upon thawing, a precipitate formed which was removed by centrifugation. The supernatant fluid was lyophilized, and this constituted the water-soluble antigens SA-S and SA-R. SA-SL was prepared similarly from living organisms.

The sediment obtained after centrifugation at $5,500 \times g$ was suspended in distilled water. It was centrifuged at $3,000 \times g$ for 15 min, and the pellet was discarded. This low-speed centrifugation was repeated three times in an attempt to remove unbroken cells. The material was finally centrifuged at $5,500 \times g$, and the pellet was washed two times in distilled water and then lyophilized. This insoluble fraction was considered a crude cell wall preparation and was designated CW-S if derived from smooth cells and CW-R if derived from rough cells.

An ether-water extract (EW) was prepared according to the method of Ribí, Milner, and Perrine (17). Its endotoxic properties have been described by Baker and Wilson (1).

The EW was treated as described by Macpherson et al. (14): 2 mg of EW per ml of 0.25 N NaOH were suspended, held for 1 hr at 56°C , neutralized with acetic acid, and centrifuged at $10,000 \times g$ for 1 hr. The supernatant fluid was precipitated with 3 volumes of ethyl alcohol, and the sediment was dialyzed and lyophilized. This preparation was designated EW-T.

The antigen employed in the agglutination test was

the U.S. Department of Agriculture standard serum tube agglutination test antigen at the recommended final concentration for diagnosis of bovine brucellosis.

Preparation of immune sera. Rabbits were hyperimmunized with water-soluble extract (SA-S) and cell walls (CW-S) from smooth *B. abortus*. A preparation containing 5 mg of lyophilized antigen per ml of incomplete Freund adjuvant (Difco) was injected subcutaneously in doses of 1 ml given three times per week for 4 weeks, followed by increased dosages three times per week for 4 more weeks, until a total of 160 mg had been injected. Two rabbits were immunized with each preparation. Rabbits were bled 1 week after the last injection. Previous studies of serum samples taken at intervals after immunization (R. Diaz, M.D. Thesis, University of Navarra, Pamplona, Spain, 1965) had shown that highest titers of agglutinins, hemagglutinins, and precipitins were obtained at this time, and no indication of inhibition of antibody production was observed at any time after this course of immunization.

Equal volumes of sera from the two rabbits immunized with each preparation were pooled before testing.

Immunological methods. The methods employed for agglutination, gel diffusion, immunoelectrophoresis, and for the indirect-hemagglutination test with tanned sheep red blood cells sensitized with water-soluble antigens (HA-1) have been described previously (8). The indirect-hemagglutination test with normal sheep red blood cells (HA-2) differed from HA-1 in the use of $100 \mu\text{g}$ of EW-T per ml of 1% normal erythrocytes.

Serum was absorbed by mixing with quantities of lyophilized antigen, placing the mixture on a shaker at 37°C for 2 hr, removing to 4°C for 18 hr, and centrifuging at $5,000 \times g$ to remove the precipitate. Various quantities of antigens were employed to determine the optimal absorbing mixture. With cell wall antigens,

30 mg per ml of serum was optimal for removal of hemagglutinins. With soluble antigens, 40 mg per ml of serum removed precipitins demonstrable by immunoelectrophoresis. With EW-T, 5 mg per ml of serum removed agglutinins. With whole bacterial cells, 0.5 ml of packed cells per ml of serum removed agglutinins. Unless otherwise stated, these were the amounts of antigen employed in the experiments presented in Tables 3 and 4.

RESULTS

Red cell sensitizing activity of EW-T and SA-S.

Table 2 shows the results when normal and tanned erythrocytes were sensitized with EW-T and SA-S for 30 or 90 min, or for both. The optimal concentration of SA-S for tanned erythrocytes was 100 μ g per ml of erythrocytes; however, when normal erythrocytes were treated with the same amount, or with 500 or 1,000 μ g of SA-S, no hemagglutination was observed. On the other hand, when normal erythrocytes were treated with EW-T, high titers were obtained in the hemagglutination test. The optimal concentration of EW-T for sensitization of normal erythrocytes was 100 μ g/ml, and 90 min of incubation was necessary for the attachment. For the attachment of SA-S to tanned cells, 30 min was sufficient. Untreated EW was not able to sensitize normal or tanned erythrocytes. EW-T was able to attach to tanned cells after 90 min of incubation, but the hemagglutination titers were low.

These results show that two antigens with different abilities to sensitize erythrocytes can be obtained from *Brucella*. Antigen SA-S sensitized cells which had been treated with tannic acid,

whereas EW-T was able to sensitize normal cells. The hemagglutination titers of the two immune sera suggest that CW-S and SA-S contain both of the antigens involved in the two hemagglutination tests, although they may be present in different proportions. Anti-SA-S serum had a higher hemagglutination titer in the test with SA-S attached to tanned erythrocytes (henceforth referred to as HA-1), whereas anti-CW-S serum had a higher hemagglutination titer in the test with EW-T attached to normal erythrocytes (designated HA-2).

Absorption of antisera. The two immune sera were absorbed with different antigens and then examined in the two indirect-hemagglutination and agglutination tests (see Table 3). Agglutinins and hemagglutinins (HA-2) were removed by absorption with all smooth antigens, but not by antigens from rough *Brucella*. Living smooth *Brucella*, EW-T and CW-S were the most efficient absorbing antigens. In the case of anti-CW-S serum, SA-SL was able to absorb more agglutinins than SA-S.

In contrast, the hemagglutinins (HA-1) were removed by antigens from both smooth and rough *Brucella* but not by living smooth or rough *Brucella* or by EW-T.

These results indicate that the agglutigen and the hemagglutigen (HA-2) are associated with the smooth *Brucella* cell wall, since their antibodies are removed by living smooth *Brucella*, and by EW-T obtained by a procedure which theoretically extracts surface antigens. On the other hand, the hemagglutigen (HA-1) is apparently not affected by smooth-to-rough variation; it must be associated with the internal structure of the *Brucella* cell, since its antibodies

TABLE 2. Indirect-hemagglutination titers obtained with sheep erythrocytes treated in various ways and tested against immune and normal sera

Preparation of erythrocytes			Indirect-hemagglutination titers obtained with antisera		
Erythrocytes	Antigen for sensitization	Time for sensitization (min)	Anti-SA-S	Anti-CW-S	Normal
Tanned	SA-S ^a	30	156,250	6,250	<50
Tanned	EW-T ^a	30	<50	<50	<50
Tanned	EW-T	90	160	640	<25
Normal	EW	90	<50	<50	<50
Normal	EW-T ^a	90	400	12,800	<50
Normal	SA-S ^a	30	<25	<25	<25
Normal	SA-S ^b	90	<25	<25	<25

^a Optimal concentration of antigen was 100 μ g per ml of erythrocytes.

^b Attempts to sensitize with 500 and 1,000 μ g per ml of erythrocytes were unsuccessful.

TABLE 3. Indirect-hemagglutination and agglutination titers obtained before and after absorption of antisera

Antigen for absorption	Titers with anti-SA-S serum			Titers with anti-CW-S serum		
	HA-1 ^a	HA-2 ^b	A ^c	HA-1	HA-2	A
None	156,250	400	200	6,250	12,800	3,200
SA-S	<50	<25	<25	<50	<25	200
SA-SL	<50	<25	<25	<50	<25	<25
SA-R	<50	400	200	<50	12,850	3,200
CW-S	<50	<25	<25	<50	<25	<25
CW-R	<50	400	200	<50	12,850	3,200
S	31,250	<25	<25	6,250	<25	<25
R	31,250	400	200	6,250	12,850	3,200
EW-T	156,250	<25	<25	6,250	<25	50

^a Indirect-hemagglutination test with tanned sheep red blood cells sensitized with SA-S.

^b Indirect-hemagglutination test with normal sheep red blood cells sensitized with EW-T.

^c Agglutination test with whole cell smooth *Brucella abortus* antigen.

cannot be removed by living smooth or rough *Brucella* cells or by EW-T, even when 20 mg of EW-T per ml of serum was employed.

SA-S and CW-S stimulate the production of antibodies to the three kinds of tests and absorb all of these antibodies. They appear to contain both surface and internal antigens.

Antigenic analysis of SA-S and SA-R by immunoelectrophoresis. The absorption experiments showed that SA-S removed the agglutinins and hemagglutinins (HA-2) from antisera, but absorption with SA-R did not remove them. We wanted to know whether immunoelectrophoresis would reveal the antigenic components in SA-S which were not present in SA-R. Anti-SA-S serum developed at least 23 precipitation lines with either SA-S or SA-R. Anti-CW-S serum developed six lines with each of the antigens. Antisera were absorbed with 50 mg of SA-S or SA-R, and all precipitins for both antigens were removed in both cases. No difference in the antigenic composition of SA-S and SA-R could be detected by immunoelectrophoresis. Figure 1 is a photograph of the immunoelectrophoresis reactions with SA-S antigen and both antisera before and after absorption with SA-R. Absorption of anti-CW-S serum with SA-R removed all precipitins (Fig. 1) but did not absorb the agglutinins or the hemagglutinins (HA-2), as shown in Table 3; therefore, we were unable to associate any of the precipitation lines produced by SA-S in immunoelectrophoresis with the agglutinin or the hemagglutinin (HA-2).

Antigenic analysis of EW and EW-T by agar-gel diffusion and immunoelectrophoresis. In a preliminary experiment, we found that EW-T did not absorb precipitins against SA-S, although

it did absorb agglutinins and hemagglutinins (HA-2). We then attempted to determine whether the precipitation techniques would reveal an antigenic component in EW-T that could be identified with the agglutinin and the hemagglutinin (HA-2), and also whether they would reveal antigenic differences between EW and EW-T.

EW developed at least eight precipitation lines in immunoelectrophoresis with anti-SA-S serum. One without electrophoretic mobility was situated around the antigen well. EW-T developed only one band situated around the antigen well in the same position as the band from EW. This band was designated X and only appeared in immunoelectrophoresis when the concentration of antigen was 50 mg/ml and a well 4 mm in diameter was used.

By the gel diffusion method, band X from EW-T cross-reacted with the similar band from EW. In the gel diffusion tests, the antigen was employed at a concentration of 25 mg/ml, as this was the optimal concentration for a clear band X. We were primarily concerned with the behavior of band X and not with the maximal number of lines which could be produced with other antigenic concentrations of EW. Absorption experiments summarized in Table 4 and illustrated in Fig. 2 show that band X was removed by absorption of serum with smooth antigens but not with rough antigens. Living smooth *Brucella* and EW-T absorbed band X but not the fast-diffusing bands, whereas living rough *Brucella* did not absorb any band. Absorption with SA-S removed all bands from anti-SA-S serum but did not remove band X or all agglutinins from anti-CW-S serum. SA-SL was able to remove all antibodies from both sera, which suggests that the specific antigen content of SA-SL must be high. SA-R was not able to absorb band X but did remove the fast-diffusing bands. Table 4 shows that in nearly all cases the removal of band X was correlated with the removal of agglutinins and hemagglutinins (HA-2).

These results indicate that the ether-water extraction of smooth *Brucella* solubilizes the surface antigen associated with the agglutinin, hemagglutinin (HA-2), and band X, but it also solubilizes other antigens in common with rough *Brucella* as shown by several lines of evidence. (i) The precipitation bands, in addition to band X, produced by EW do not appear when reacted against serum that has been absorbed with SA-R. (ii) Anti-rough *Brucella* serum will develop these additional bands but will not develop band X. Attempts were made to develop band X with SA-S and SA-SL as antigens in the agar-gel diffusion test, but these were unsuccessful. This

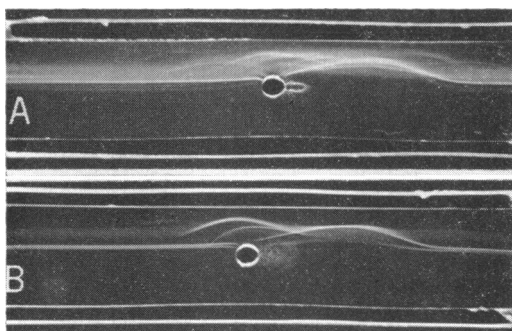


FIG. 1. Immunoelectrophoretic pattern of antigen SA-S (in wells) developed by (A) anti-SA-S serum unabsorbed (upper trough) and absorbed with SA-R antigen (lower trough) and (B) anti-CW-S serum unabsorbed (upper trough) and absorbed with SA-R antigen (lower trough).

TABLE 4. Correlation of absorption of precipitin associated with band X with absorption of hemagglutinins (HA-2) and agglutinins

Antigen for absorption	Results with anti-SA-S serum					Results with anti-CW-S serum						
	Bands developed in gel diffusion test with EW antigen				Titer with HA-2 ^a	Titer with A ^b	Bands developed in gel diffusion test with EW antigen				Titer with HA-2	Titer with A
	X	1	2	3			X	1	2	3		
None	+	+	+	+	400	200	+	+	+	+	12,800	3,200
SA-S	-	-	-	-	<25	<25	+	-	-	-	<25	200
SA-SL	-	-	-	-	<25	<25	-	-	-	-	<25	<25
SA-R	+	-	-	-	400	200	+	-	-	-	12,800	3,200
S	-	+	+	+	<25	<25	-	+	+	+	<25	<25
R	+	+	+	+	400	200	+	+	+	+	12,800	3,200
EW-T	-	+	+	+	<25	<25	-	+	+	+	<25	50

^a Indirect-hemagglutination test with normal sheep red blood cells sensitized with EW-T.

^b Agglutination test with whole cell smooth *Brucella abortus* antigen.

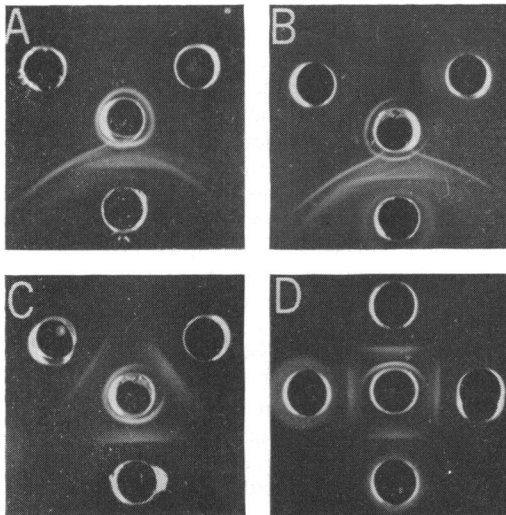


FIG. 2. Agar gel diffusion patterns obtained with antigen EW in center well in all patterns. Surrounding wells, beginning with upper left-hand corner, contained (A) anti-CW-S serum absorbed with SA-R, absorbed with SA-S, and unabsorbed, (B) anti-CW-S serum absorbed with SA-R, absorbed with SA-SL, and unabsorbed, (C) anti-CW-S serum absorbed with living rough *Brucella*, absorbed with living smooth *Brucella*, and unabsorbed, (D) anti-CW-S serum absorbed with EW-T in the proportion of 500 μ g, 1 mg, 5 mg, and 10 mg per ml of serum, respectively.

means that the antigen corresponding to band X must be in some nondiffusible form in SA-S. The antigen must be present, since SA-S is able to remove the precipitin to band X in absorption experiments. The antigen corresponding to band

X is present on smooth but not rough *Brucella* cells; it appears to be situated on the surface of the cell wall, as living smooth *Brucella* cells are able to absorb its antibody. The correlation between the absorption of band X and the absorption of agglutinins and hemagglutinins (HA-2) suggests that a single antigen is specific for the three immunological reactions.

DISCUSSION

These studies indicate that two kinds of substances, which differ in their capacity for sensitizing sheep red blood cells, can be isolated from *B. abortus*. The antigens contained in the treated ether-water extract (EW-T) were able to sensitize normal erythrocytes. Keogh et al. (12) showed that the bacterial antigens which sensitized normal erythrocytes were polysaccharides. Some polysaccharides, however, were unable to attach to normal cells until the antigens were treated with dilute alkali (14). We found that EW from *Brucella* cells was unable to sensitize normal cells until it was treated with NaOH.

The antigen contained in the water-soluble extract (SA-S) was able to sensitize tanned red blood cells, but not normal red cells. Boyden (5) showed that pretreatment of red blood cells with tannic acid was usually required for the attachment of protein antigens. The protein concentration of the water-soluble antigens was found to be from 62 to 75% (8).

Sulitzeanu (20) was unable to correlate the agglutinin titer of a *Brucella* antiserum with its ability to produce lines in agar, nor was loss of precipitation lines correlated with loss of agglutinins. We also found this to be true with water-soluble antigens. In the immunoelectrophoretic

analysis of SA-S, no band of precipitation could be correlated with the agglutinin or hemagglutinin (HA-2). In the case of antigen EW, however, the precipitation band X could be correlated with the agglutinin and hemagglutinin (HA-2). This band X was absorbed by smooth antigens but not by rough antigens. EW-T, which developed only the precipitation band X, could not absorb any precipitins developed by SA-S but could absorb agglutinins and hemagglutinins (HA-2). Purification of antigen EW-T is in progress to see whether a single antigen determinant carries the specificity for agglutinins, hemagglutinins (HA-2), and band X production.

Although both kinds of red cell-sensitizing substances could be isolated from smooth *Brucella*, only the water-soluble extract able to sensitize tanned cells was isolated from rough *Brucella*. Baker and Wilson (1) were unable to extract endotoxin from rough *B. abortus*. We were also unsuccessful in extracting a polysaccharide type of antigen from rough *Brucella* by ether-water or trichloroacetic acid treatment. Dubrovskaya (9) extracted antigen from rough *B. melitensis* by both the trichloroacetic acid and the phenol-water methods, although the yield was approximately half of that obtained from smooth *B. melitensis*.

It was not possible to demonstrate a clear difference in the origin of SA-S and CW-S antigens. SA-SL contained antigenic determinants capable of absorbing agglutinins, both hemagglutinins, and the precipitin corresponding to band X from its homologous antiserum, and it absorbed both hemagglutinins from anti-CW-S serum. CW-S was able to absorb agglutinins and both hemagglutinins from its own antiserum and from anti-SA-S. It is possible that the crude cell wall preparation (CW-S) contained some soluble antigen. It is also likely that ultrasonic treatment solubilized cell wall material.

Ether-extraction theoretically removed only the surface antigen from smooth cells, but EW developed precipitation lines, in addition to band X, which were related to rough antigens, as the precipitins for these additional lines could be removed by absorption of the serum with SA-R.

The difficulty in clearly separating materials from cell walls and protoplasm has been encountered by other workers (10, 15, 19, 21).

Markenson et al. (15) reported that ultrasonic treatment of *Brucella* produced small particles of cell wall that remained in suspension during centrifugation at 10,000 rev/min, but were sedimented at 40,000 rev/min. The pellet resulting from centrifugation at 40,000 rev/min contained diaminopimelic acid, a substance believed to be from the cell wall. We observed that SA-SL had

more capacity for absorbing agglutinins than SA-S. It is probable that ultrasonic treatment of living *Brucella* results in greater disintegration of the cell wall. Ribí et al. (17) found that living bacteria were broken more efficiently than ether-treated bacteria.

Glenchur et al. (10) studied the components of mechanically disrupted *B. melitensis* organisms obtained by differential centrifugation and diethylaminoethyl cellulose ion-exchange chromatography. Nearly every fraction or component was capable of stimulating production of agglutinins and precipitins, which could respectively agglutinate whole cells and produce precipitation bands to soluble antigens, as shown by agar-gel diffusion and immunoelectrophoresis.

Baughn and Freeman (3) reported that antigens prepared by ultrasonic treatment of living *B. suis* revealed four major and nine minor precipitating antigens in immunoelectrophoretic analysis. After absorption with packed Formalin-killed *B. suis*, the serum no longer developed lines associated with four of the minor antigens. They were believed to be associated with the cell wall surface. These four antigens were not detected in the immunoelectrophoretic analysis of spheroplast antigen preparations of *B. suis*. These results are in contrast to the findings of Olitzki and Sulitzeanu (16) and Diaz (M.D. Thesis, University of Navarra, Pamplona, Spain, 1965), who found that living whole *Brucella* were not able to absorb precipitating antibodies. It is possible that the surface antigens of living *Brucella* prevent antibody contact with other surface antigen determinants. Formalin treatment used by Baughn and Freeman (3) could possibly remove some of the surface antigens, thus exposing other antigenic components to absorb antibodies. It is also possible that Formalin killing causes cell breakage or leakage, thus releasing soluble antigens.

Diaz and Chordi (7) showed that an ether-water extract from *B. suis* developed nine precipitation lines with its homologous hyperimmune serum. A line lacking electrophoretic mobility, similar to band X in EW of the present study, was not observed, probably because insufficient antigen concentrations were employed in immunoelectrophoresis. All the lines from ether-water antigen showed cross-reactions with lines from water-soluble antigens.

Hinsdill and Berman (11) observed nine precipitation lines in immunoelectrophoretic analysis of water-soluble antigens obtained from ultrasonically treated *B. abortus*. The ninth antigenic component formed a diffuse heavy precipitate inside the antigen well. Absorption of the immune serum with phenol-killed *B. abortus* re-

moved the precipitin for the ninth component only, suggesting that it was of cell surface origin. A similar component was observed in phenol extracts which after partial acid hydrolysis formed a line just outside the antigen well. The lack of electrophoretic mobility of this component, and its absorption by whole cells is similar to the behavior of band X of the present study.

Widely different methods for antigen preparation, immunization, and immunological analysis make interpretation of results difficult. Further work on the separation and purification of these antigens is necessary to determine which are responsible for the development of protective immunity.

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