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Isolation of Components of *Brucella abortus* Responsible for Inhibition of Function in Bovine Neutrophils

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The effects of fractions of *Brucella abortus* strain 2308 on functions of bovine polymorphonuclear neutrophils (PMNs) were examined in vitro. Ingestion of *Staphylococcus aureus* and reduction of nitroblue tetrazolium dye by bovine PMNs were not inhibited by heat-killed *B. abortus*. The ability of PMNs to iodinate proteins was significantly inhibited by live or heat-killed *B. abortus* and supernatant from heat-killed cells but not by washed heat-killed cells. Two inhibitory components isolated from the supernatant by high-performance liquid chromatography were characterized as nucleotide-like substances with molecular weights of <1,000. Inhibition of iodination by these components was concentration dependent. These results indicate that one of the mechanisms by which *B. abortus* may escape intracellular killing by PMNs is through the production of low-molecular-weight components that inhibit the myeloperoxidase–hydrogen peroxide–halide antibacterial system of bovine PMNs.

Bovine brucellosis continues to be a disease of major economic significance in North America and throughout the world. The causative agent, *Brucella abortus*, is a facultative intracellular parasite capable of surviving within PMNs and nonactivated macrophages [1, 2]. The mechanisms and virulence factors responsible for evasion of the host’s phagocytic system are not well understood. Smith [3] hypothesized that a cell wall component of *B. abortus* interfered with the bactericidal mechanisms of phagocytes. Virulent *B. abortus* from guinea pig monocytes or infected bovine placenta had enhanced ability to survive intracellularly compared with the same strain grown on laboratory medium. Cell wall material of organisms from infected bovine placenta inhibited intracellular destruction of an attenuated strain of *B. abortus* [4]. The inhibitory activity for PMNs was not present in cell wall material from organisms grown in vitro unless bovine allantoic fluids were present in the culture medium [3, 5].

Kreutzer et al. [6] and Riley and Robertson [7] compared the ability of human and bovine PMNs to ingest and kill smooth and rough strains of *B. abortus*. Both bacterial strains were readily ingested by either human or bovine phagocytes. Both smooth and rough strains were resistant to killing by human and bovine PMNs; however, the smooth strain was more resistant than the rough strain. Although *B. abortus* resisted intracellular killing, some of the organisms were killed. By electron microscopy they showed that degranulation of both primary and secondary PMN granules was inhibited by *B. abortus* and that viable organisms were not required to inhibit degranulation. Thus they hypothesized that there may be a unique surface component responsible for the inhibition of degranulation by PMNs that allowed the bacteria to resist bactericidal activity by PMNs.

It was shown by using granule extracts that the myeloperoxidase (MPO)-H₂O₂-halide system appeared to be effective in killing *Brucella* if the system was allowed to function normally [8]. Thus inhibition of degranulation would appear to be an important factor in the intracellular survival of *B. abortus*. Kreutzer and Robertson [9] indicated that the component responsible for this activity may have been a toxic lipopolysaccharide. Some studies [9, 10] have also indicated that ingestion of *B. abortus* did not stimulate the oxidative metabolic burst that normally accompanies ingestion; thus, the H₂O₂ required by the MPO-H₂O₂-halide antibacterial system may not have been produced. *B. abortus*
did not inhibit the oxidative metabolic burst if neutrophils were stimulated with a different bacterium [9].

The purpose of this study was to determine the effects of virulent \textit{B. abortus} on specific aspects of bovine PMN function. In vitro assays were conducted in the presence of \textit{B. abortus} (or fractions of the organism) to determine the ability of PMNs to carry out ingestion, the metabolic oxidative burst, and the MPO-H$_2$O$_2$-halide reaction. In addition, factors inhibitory to PMNs that were produced by \textit{B. abortus} were isolated and partially characterized.

**Materials and Methods**

**Bacterial preparation.** Strain 2308 of \textit{B. abortus}, a highly virulent, smooth form of bovine origin, was used throughout this study. Cultures were inoculated into a fermentor containing 12 liters of medium composed of Bacto-dextrose (30 g/liter), peptone “M” (30 g/liter), Bacto-yeast extract (10 g/liter; all from Difco, Detroit), Na$_2$HPO$_4$ (9 g/liter), Na$_2$HPO$_4$ (3.3 g/liter), and distilled water. The culture was supplied with a source of filter-sterilized air. After incubation for 48 hr at 37 C, the cells were harvested by centrifugation at 18,000 g for 20 min. The bacteria were then resuspended in 0.85% NaCl and standardized turbidimetrically to a concentration of \(4 \times 10^{10}\) cells/ml. A portion of the standardized bacteria was used directly in the assays of PMN function, and the remaining cells were heat killed at 65 C for 1 hr. An aliquot of these cells was cultured for three days to ensure that no viable cells remained.

**Bacterial fractions.** A portion of the standardized heat-killed bacterial suspension was saved and used as the whole heat-killed cell fraction. The remainder was centrifuged at 18,000 g for 20 min. The pelleted cells were washed three times with PBS solution (0.15 \(M\) NaCl and 50 mM phosphate, pH 7.2) and resuspended to the original volume in PBS for use as the washed-cell fraction. A portion of the supernatant from the heat-killed cells was used as the crude supernatant fraction. The remaining supernatant was separated by molecular filtration into fractions with molecular weights of \(>10,000\) and \(<1,000\) by using PM10 and YM2 filters (Amicon Corp., Lexington, Mass), respectively. The retentate from the PM10 filtration was washed three times by resuspension to 20 ml and refiltering to 2 ml. After the final wash the retentate was resuspended to the original volume for use as the \(>1,000\)-dalton fraction. Alternatively, the filtrate from the YM2 filtration was designated as the \(<1,000\)-dalton fraction.

**Release of inhibitors by live \textit{Brucella}.** To determine whether live \textit{B. abortus} elaborates substances inhibitory for PMNs into its surroundings, we evaluated medium in which the cells had been incubated for inhibition of normal function of PMNs. In brief, \textit{B. abortus} cells were grown to a density of \(8 \times 10^{10}\) cells/ml in a fermentor as described above. The bacteria were washed three times with 0.85% NaCl and resuspended in Earle's balanced salt solution (Gibco, Grand Island, NY) to a concentration of \(4 \times 10^{10}\) cells/ml. The cells were incubated on a rotary shaker for \(\sim 26\) hr at 37 C, after which the bacteria were pelleted by centrifugation at 18,000 g for 20 min. The culture supernatant was filtered twice through filters (pore size, 0.45 \(\mu m\)) and checked for sterility. The filtrate was then passed through a YM2 filter as described above. The filtrate was designated as the culture supernatant fraction.

**High-performance liquid chromatography (HPLC).** Further separation of the \(<1,000\)-dalton fraction was performed with reverse-phase HPLC. In brief, 1-ml samples were injected onto a reverse-phase \(\mu\)Bondapack$^\text{®}$ C$_{18}$ column (7.8 x 300 mm; Waters Associates, Milford, Mass). The mobile phase consisted of a solution of 5% methanol and 0.1% ammonium bicarbonate in water adjusted to pH 6.4 with 1 N HCl. The flow rate during the separation was 1.5 ml/min, and the effluent was monitored at a wavelength of 254 nm. Individual peaks were collected and dried in a lyophilizer to remove the volatile solvent components. After drying, the fractions were resuspended to their injection volume. HPLC fraction 3 was rechromatographed with the same solvent and column at a flow rate of 1.0 ml/min to obtain better separation of the components of interest.

**Characterization of fractions.** Several procedures were used to characterize physically the \(<1,000\)-dalton fraction. To determine whether the material was heat stable, we autoclaved an aliquot of the fraction for 15 min at 121 C before the material was assayed for biologic activity. This material was designated as the autoclaved fraction. The pH stability of the material was determined by addition of 0.1 ml of either 1 N HCl or 1 N NaOH to alter the pH of the sample (0.1 ml) to pH 2.3 or 12.0, respectively. After incubation for 30 min the samples were neutralized to pH 7.2 by addition of either 1 N HCl or 1 N NaOH. The samples were then compared with...
nontreated volume-matched samples of the <1,000-dalton fraction for their ability to inhibit function of bovine PMNs.

Partial chemical characterization of biologically active HPLC-purified fractions was obtained by a variety of procedures. Protein content was determined by the Folin phenol method [11] with bovine serum albumin as the standard. Total carbohydrate content was determined with the phenol-sulfuric acid method as described by Dubois et al. [12]. The wavelength of maximum absorbance was identified with use of a scanning ultraviolet spectrophotometer.

Further structural characterization was obtained by thin-layer chromatography. In brief, 50-μl samples were spotted onto silica gel plates (silica gel G; Analtech, Newark, Del). The mobile phase consisted of a solution of 70% propanol in water. Rhodamine 6G was used for the detection of lipids [13]. Iodine vapor was used to identify unsaturation [13]. A solution of 0.3% H2O2 was used to detect aromatic acids, and ninhydrin was used to identify primary amino groups [13].

Preparation of PMNs. Twenty apparently healthy adult Holstein-Friesian steers, serologically free of infectious bovine rhinotracheitis virus and bovine viral diarrhea virus, were used as a source of PMNs throughout the course of this study. These animals were housed together in outdoor pens and fed grain and hay once daily. None of the animals had any known previous exposure to B. abortus. Bovine PMNs were isolated as previously described [14]. In brief, peripheral blood was collected in acid citrate-dextrose solution and centrifuged at 18,000 g for 20 min. The resulting plasma and Buffy coat cells were discarded. Erythrocytes present in the packed cell layer were lysed with phosphate-buffered deionized water, and the remaining cells, which generally consisted of >90% granulocytes, were washed and resuspended in PBS to a concentration of 5 × 106 cells/ml. The cells were held at room temperature (∼23 C) for ∼1 hr before use in all three assays of PMN function.

Tests of PMN function. Tests of PMN function [14] were conducted in duplicate, and the average of duplicate values was used for calculation.

Ingestion of Staphylococcus aureus. Heat-killed S. aureus labeled with [125I]iododeoxyuridine (Amer­sham Corp., Arlington Heights, Ill) was used to evaluate ingestion by PMNs. The test was performed in plastic tubes (12 × 75 mm), and the standard reaction mixture contained 0.1 ml of [125I]iododeoxy­uride-labeled S. aureus, 0.05 ml of PMNs (2.5 × 106 PMNs; bacteria/PMN ratio, 60:1), 0.05 ml of a 1:10 dilution of bovine antisem to S. aureus, and 0.3 ml of Earle's balanced salt solution. To determine the effects of bacterial fractions on ingestion by PMNs, we added 0.1 ml of a B. abortus fraction (4 × 106 cells or equivalent fraction) or 0.1 ml of PBS as a control to the standard reaction mixture. The reaction mixture was incubated for 10 min at 37 C, after which extracellular S. aureus organisms were removed by treatment with lysozyme. The PMNs were washed by centrifugation at 1,800 g for 10 min, and the amount of PMN-associated radioactivity was determined. Results were expressed as percentages of labeled S. aureus that were ingested.

Reduction of nitroblue tetrazolium (NBT) dye. Reduction of NBT measures the normal oxidative metabolic burst of PMNs. The assay was conducted in siliconized glass tubes (15 × 100 mm), and the standard reaction mixture contained 0.2 ml of NBT solution (2 mg/ml), 5 × 106 PMNs, 0.1 ml of preopsonized zymosan preparation (10 mg/ml), and 0.6 ml of Earle's balanced salt solution. To determine the effects of bacterial fractions on reduction of NBT by PMNs, we added 0.2 ml of a B. abortus fraction (8 × 106 cells or an equivalent fraction) or 0.2 ml of PBS as a control to the standard reaction mixture. After incubation for 10 min at 37 C, the reaction was stopped. Purple formazan, formed as a product of reduction of NBT, was extracted with pyridine, and the OD580 was determined. Results were expressed as the OD580 × 106 PMNs per 10 min in 5.0 ml of pyridine.

Iodination assay. The iodination assay (a measure of the MPO-H2O2-halide activity of PMNs) was performed in capped polystyrene tubes (12 × 75 mm). The standard reaction mixture contained 0.05 ml of PMNs (2.5 × 106 cells), 0.05 μCi of 125I (carrier-free; Amersham), 40 nmol of NaI, 0.05 ml of preopsonized zymosan (10 mg/ml), and 0.3 ml of Earle's balanced salt solution. To determine the effects of bacterial fractions on iodination by PMNs, we added 0.1 ml of a B. abortus fraction (4 × 106 cells or an equivalent fraction) or 0.1 ml of PBS as a control to the standard reaction mixture. The reaction was allowed to proceed for 20 min at 37 C before the amount of trichloroacetic acid–precipitable radioactivity was determined. Results were expressed as nmol of NaI/106 PMNs per hour.

To determine the effects of bacterial fractions on each PMN function, we compared the value obtained
when a bacterial fraction was added to PMNs with the value obtained with control (PBS-treated) PMNs from the same animal. Either an analysis of variance procedure (blocked by day) or Student's t test was used to determine significance of the differences in PMN function. For data presentation all treatment values are expressed as percentages of control values, defined as (experimental value/control value) × 100.

Reversibility of effects and masking studies. To determine whether inhibitory activities seen in iodination studies were reversible, we preincubated PMNs (2.5 × 10⁶ cells) for 20 min with the <1,000-dalton fraction (0.1 ml) and washed them three times with PBS before addition to the normal iodination reaction mixture. To determine whether the inhibitory substance bound to the preopsonized zymosan and prevented its stimulation of PMNs, we preincubated 0.05 ml of preopsonized zymosan with 0.1 ml of the <1,000-dalton fraction for 20 min. After preincubation and three washes with PBS, the treated zymosan was added to the normal reaction mixture for use in the iodination assay.

Xanthine-xanthine oxidase-horseradish peroxidase-mediated iodination. To study further the effects of the <1,000-dalton fraction on iodination by PMNs, we used an enzyme-catalyzed iodination reaction that did not involve PMNs. The procedure used has been described previously [15]. In brief, xanthine oxidase and xanthine (Sigma Chemical Co., St. Louis) were used to catalyze the formation of superoxide anion. The H₂O₂ formed from the superoxide anion then served as the substrate for horseradish peroxidase (Sigma), used to catalyze the iodination of endogenous proteins. The standard reaction mixture contained 40 nmol of NaI, 0.05 μCi of ¹²⁵I, 0.3 ml of Earle's balanced salt solution containing 0.1% bovine serum albumin, 0.5 mg of xanthine, 0.5 U of horseradish peroxidase, and 0.1 ml of the <1,000-dalton fraction or PBS as a control. The reaction was started by addition of 0.02 U of xanthine oxidase, after which the mixture was incubated and processed by the same procedures as those used for iodination by PMNs. A blank containing all of the reaction components except xanthine oxidase was assayed with each experiment. The results are expressed in cpm.

Results

Effect of fractions of B. abortus on ingestion of S. aureus. The results of studies of the ingestion of S. aureus are shown in table 1. Ingestion of bacteria was inhibited (66.0% ± 6.2% of control values) significantly (P < .01) by the washed-cell fraction. The whole heat-killed cell fraction was also inhibitory (84.4% ± 7.1% of control values); however, this suppression was not statistically significant (P > .05). No other fractions had any significant (P > .05) effect on the phagocytic ability of bovine PMNs.

Effect of fractions of B. abortus on reduction of NBT. The results of the studies of reduction of NBT are presented in table 1. None of the bacterial fractions tested had any significant (P > .05) effect on the ability of PMNs to produce superoxide anion as measured by reduction of NBT.

Effect of fractions of B. abortus on iodination. The effects of the crude bacterial fractions on the ability of PMNs to iodinate protein are presented in table 1. Iodination of proteins by PMNs was significantly inhibited (P < .01) by live Brucella (34.1% of control values). In addition, the iodination ability of PMNs was significantly (P < .01) inhibited 38%–54% in the presence of whole heat-killed cells, supernatant, and the <1,000-dalton fraction. The washed-cell, live cell supernatant, and >10,000-dal-

| Table 1. Effects of fractions of B. abortus on in vitro functions of PMNs. |
|--------------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| PMN function assay | Live B. abortus strain 2308 | Whole heat-killed cells | Live cell culture supernatant | Supernatant from killed cells | Washed cells | <1,000 daltons | >10,000 daltons |
| S. aureus ingestion | ND               | 84.8 ± 7.1       | 104.7 ± 5.4     | 97.5 ± 8.4       | 66.0 ± 6.2*     | 114.4 ± 8.6    | 91.0 ± 9.4      |
| NBT reduction      | ND               | 101.5 ± 15.2     | 98.6 ± 7.3      | 95.5 ± 12.8      | 93.4 ± 9.5      | 103.1 ± 13.7   | 92.1 ± 6.6      |
| Iodination         | 34.1 ± 1.7*     | 45.6 ± 1.8*      | 109.5 ± 8.3     | 60.9 ± 5.7*      | 98.5 ± 7.7      | 61.7 ± 3.4*    | 93.1 ± 7.0      |

NOTE. Data are mean ± SE percentages of control values (n = 6). ND = not done.
* P < .01 by analysis of variance compared with control (PBS-treated) cells.
B. abortus Inhibition of Neutrophil Function

Effects of pH- and heat-treated <1,000-dalton fraction and pretreated opsonized zymosan and PMNs on iodination by bovine PMNs. Data are mean ± SE (bars) values (n = 6). MW = molecular weight. * P < .01.

Figure 1. Effects of pH- and heat-treated <1,000-dalton fraction and pretreated opsonized zymosan and PMNs on iodination by bovine PMNs. Data are mean ± SE (bars) values (n = 6). MW = molecular weight. * P < .01.

Table 2. Effect of the <1,000-dalton fraction of B. abortus on xanthine-xanthine oxidase-horseradish peroxidase-mediated iodination.

<table>
<thead>
<tr>
<th>Addition</th>
<th>Iodination (cpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank (no xanthine oxidase)</td>
<td>313 ± 63</td>
</tr>
<tr>
<td>Control (PBS)</td>
<td>1,259 ± 76</td>
</tr>
<tr>
<td>&lt;1,000-dalton fractions</td>
<td>1,273 ± 50</td>
</tr>
</tbody>
</table>

NOTE. Data are mean ± SE values (n = 5).

Figure 2. Primary separation of the <1,000-dalton fraction by reverse-phase HPLC.

Effects of the <1,000-dalton fraction on xanthine-xanthine oxidase-horseradish peroxidase-mediated iodination. The effects of the <1,000-dalton fraction on an enzyme-catalyzed iodination reaction that did not involve PMNs were examined. This procedure involved the use of xanthine oxidase and xanthine to catalyze the formation of superoxide anion. H2O2 formed from the superoxide anion then served as the substrate for horseradish peroxidase, which was used to catalyze the iodination of proteins. The <1,000-dalton fraction did not inhibit the xanthine-xanthine oxidase-horseradish peroxidase iodination system (table 2).

Results of HPLC separation of the <1,000-dalton fraction. The results of the first reverse-phase separation of the <1,000-dalton fraction are shown in figure 2. Results of testing each of the fractions labeled in figure 2 on iodination indicated that the inhibitory activity for PMNs was contained in peaks 3 and 10. Peak 3 was re-injected onto the column, and the separation obtained is shown in figure 3. The effects of all HPLC-separated fractions on iodination by PMNs are presented in figure 4. Iodination was significantly (P < .01) inhibited by fraction 3b, fraction 10 (P < .05), and the unseparated <1,000-dalton fraction (P < .01). None of the other fractions obtained by HPLC had any significant effect on iodination by PMNs.

Titration of the inhibitory activity and chemical characterization of HPLC fractions 3b and 10. HPLC fractions 3b and 10 were evaluated for their
Figure 3. Separation of reinjected HPLC fraction 3 by reverse-phase HPLC.

concentration effects on iodination by bovine PMNs. The inhibitory activities expressed by both fractions were concentration dependent, but neither fraction completely inhibited the iodination reaction, even at the highest concentration tested. The results were examined by least squares analysis and expressed as percentages of control values (figure 5).

Results of protein determinations indicated that neither fraction 3b nor 10 contained measurable protein. Fraction 3b contained ~39.8 μg of carbohydrate/ml, whereas fraction 10 contained no detectable carbohydrate. Dry weight determinations indicated that HPLC fraction 3b contained ~0.156 mg of material/ml, whereas fraction 10 contained ~0.17 mg of material/ml.

The results of the thin-layer chromatography studies are shown in figure 6. When exposed to short-wave ultraviolet light, fraction 3b produced two fluorescent spots, one with an Rf of 0.31 and the other of 0.47. Fraction 10, when treated similarly, produced one spot with an Rf of 0.47. Both fractions reacted with iodine vapor, a result indicating the presence of unsaturated carbon-carbon bonds. Neither fraction contained any detectable lipids, primary amino groups, or aromatic acids.

The results of an ultraviolet light scan of the fractions indicated that both molecules exhibited maximum absorbance values at 260 nm.

Discussion

There are two major microbicidal activities that occur inside a PMN when an organism is ingested: (1) the generation of toxic products of oxygen resulting from oxidative metabolism and (2) the enzymatic destruction of the microorganism by the lysosomal enzymes present in the granules that fuse with the phagocytic vacuoles [16]. Many pathogenic organisms have developed mechanisms of resisting and inhibiting the activity of phagocytic cells and thereby facilitating their survival within the host [17].

The first step of the bacterium-PMN interaction is ingestion. Results of the assay for ingestion of *S. aureus* (table 1) indicate that this strain of *B. abortus* does not appear to produce any substances that inhibit the phagocytic ability of PMNs. The decreased ingestion of *S. aureus* seen with the washed-cell and whole heat-killed fractions was probably because of competitive inhibition of the uptake of *S. aureus* between the two bacterial species. When soluble fractions of *B. abortus* were added to the reaction mixture, no inhibition of ingestion was seen.

Figure 4. Effects of all HPLC-separated fractions on iodination by bovine PMNs. Data are mean ± SE (bars) values (n = 11). *P < .05; **P < .01.
Oxidative metabolism by the PMN is an important prerequisite for oxygen-dependent bactericidal activities. When a PMN is stimulated, the oxidase enzyme on the surface of the plasma membrane catalyzes the conversion of oxygen to superoxide anion, which is then spontaneously converted to \( \text{H}_2\text{O}_2 \). NBT is directly reduced by superoxide anion to insoluble purple formazan. Reduction of NBT is therefore a measure of production of superoxide anion by the PMN [18]. Results of the assay of NBT reduction (table 1) indicate that neither whole heat-killed cells nor fractions of \( B. \text{abortus} \) inhibit production of superoxide anion by bovine PMNs. In addition, filtered culture supernatant from live \( B. \text{abortus} \) cells did not suppress the ability of the PMNs to produce superoxide anion. Previous workers have determined that ingestion of \( B. \text{abortus} \) does not stimulate the respiratory burst in PMNs [9, 10]. Their results also indicated that if the respiratory burst was stimulated by another phagocytosable particle, \( B. \text{abortus} \) did not inhibit the oxidative burst [9]. The current work evaluated the ability of PMNs metabolically stimulated with opsonized zymosan to carry out the respiratory burst in the presence of whole or fractionated \( B. \text{abortus} \). The assay results indicate that once the respiratory burst is stimulated, the bacteria do not prevent the formation of superoxide anion.

The iodination reaction of PMNs is dependent on the generation of \( \text{H}_2\text{O}_2 \), degranulation of primary granules to release MPO, the presence of iodine, the unimpaired ability of MPO to catalyze the iodination of exposed tyrosine on proteins, and the presence of tyrosine. This system exhibits potent antimicrobial activity [19]. The results of the assays of iodination by PMNs (table 1 and figures 1 and 4) indicate that the ability of PMNs to iodinate proteins was inhibited by live \( B. \text{abortus} \), whole heat-killed \( B. \text{abortus} \), and the following fractions: crude supernatant, <1,000-dalton material, autoclaved supernatant, and low and high pH-treated <1,000-dalton material. Thus the components responsible for this inhibitory activity are heat- and pH-stable materials from the supernatant of heat-killed

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**Figure 5.** Effects of various concentrations of HPLC fractions 3b (○) and 10 (□) on iodination by bovine PMNs (\( n = 4 \)).

**Figure 6.** Thin-layer chromatographic separation of HPLC fractions 3b and 10 exposed to short-wave ultraviolet light for detection. \( R_f \) = ratio of fronts.
B. abortus that have molecular weights of <1,000. The inhibitory materials can be isolated in HPLC fractions 3b and 10, with fraction 3b being significantly \((P < .05)\) more suppressive than fraction 10 (68.4% vs. 84.5%). The results of the titration studies (figure 5) indicate that this difference may be due to a difference in the relative concentrations of these inhibitory components extracted from the bacterial cells. The purified materials were maintained at the same relative concentration as the crude material extracted from the bacterial surface, thus allowing a rough determination of percent recovery directly from the percent inhibition of iodination. Because the suppression is dose dependent and the purified materials show the same level of inhibition as the crude materials, we can assume a high rate of recovery.

Results of the iodination assays that involved the pretreated opsonized zymosan and pretreated PMNs (figure 1) indicate that the inhibitory substances do not exert their inhibition by masking the stimulatory sites on the opsonized zymosan particles and that the inhibitory effects were reversible.

Results of the iodination assays involving the culture supernatant from live Brucella cells indicate that the organism does not release detectable amounts of the inhibitory materials into the environment as a result of bacterial metabolic processes. It should be noted that these results are valid for the conditions used and that actively growing cells may behave differently. This finding indicates that the suppressive materials are an integral part of the cell surface, as suggested by other researchers [4, 7].

These results indicate that strain 2308 of B. abortus produces low-molecular-weight substances that specifically inhibit the MPO-H\(_2\)O\(_2\)-halide antibacterial system of bovine PMNs. It is possible that the inhibition of the MPO-H\(_2\)O\(_2\)-halide system is due to decreased availability of H\(_2\)O\(_2\), or a decrease in the capability of MPO to catalyze the iodination reaction. However, because the xanthine-xanthine oxidase–horseradish peroxidase–mediated iodination was not inhibited by the <1,000-dalton fraction (table 2), the rate of formation of H\(_2\)O\(_2\) from superoxide anion and the rate of destruction of H\(_2\)O\(_2\) were apparently not affected. In addition, the ability of the peroxidase enzyme to catalyze the reaction was apparently unimpaired. MPO and horseradish peroxidase are different enzymes, and it is possible that the inhibitory factors could inhibit MPO directly but not inhibit horseradish peroxidase.

Rhodococcus equi, another bacterium that resists killing by PMNs, also produces low-molecular-weight components that inhibit the MPO-H\(_2\)O\(_2\)-halide antibacterial system [20].

Previous research involving the isolation and characterization of lysosomal enzymes has indicated that B. abortus inhibits degranulation of both primary and secondary granules [6, 7]. Frenchick et al. [21] have shown that soluble extracts of B. abortus inhibit phagosome-lysosome fusion in murine peritoneal macrophages. Morphometric analysis of transmission electron micrographs of bovine PMNs treated with the <1,000-dalton fraction has indicated that this material inhibits degranulation of PMN granules (T. A. Bertram, P. C. Canning, and J. A. Roth, unpublished observation). Because degranulation is required for the release of MPO, it seems likely that fractions 3b and 10 inhibit the MPO-H\(_2\)O\(_2\)-halide antibacterial system of PMNs by preventing degranulation and the subsequent release of MPO. Smith [3] and Fitzgeorge and Smith [5] reported that B. abortus grown in vitro did not inhibit PMNs unless bovine allantoic fluids were contained within the culture medium. Our results indicate that a substance inhibitory for PMNs is produced by B. abortus strain 2308 cells grown in vitro even if bovine allantoic fluids are not present in the culture medium. This apparent discrepancy may have been due to the different strains of the organism that were used, the different methods of culturing the bacteria, or the different methods used for the detection of inhibition of PMNs.

Results of the thin-layer chromatography studies (figure 6) indicate that fraction 3b is composed of two portions. One portion comigrates with fraction 10, a result indicating that fraction 10 may be a breakdown product of fraction 3b. A summary of the characterization results indicates that neither fraction 3b nor 10 contains detectable proteins or lipids. Fraction 3b contains a small amount of carbohydrate, whereas fraction 10 contains no detectable amount. Both fractions exhibit maximum absorbance of ultraviolet light at 260 nm and are small. These results are consistent with the properties of nucleotides or nucleotide-like substances.

These studies indicate that one of the mechanisms by which B. abortus may escape intracellular killing by PMNs is through the production of low-molecular-weight components that inhibit the MPO-H\(_2\)O\(_2\)-halide antibacterial system of bovine PMNs by preventing the degranulation of PMN granules.
Because of their small size, it seems likely that these components are not immunogenic and escape detection by the bovine immune system while facilitating the intracellular survival of *B. abortus*.

**References**