Protective antigens were separated from a 2.5% saline extract of Pasteurella multocida type 4 by chromatographic methods. The organism was grown for 18 to 20 hours at 37°C on an agar medium containing 5% heat-inactivated turkey serum. The bacterial growth was harvested in a 2.5% NaCl solution and agitated for 1 hour at 56°C. The suspension was centrifuged, and the supernatant was dialyzed for 72 hours against 0.85% NaCl solution (crude extract:CE). In gel filtration with a Sephadex G-200 column two protein peaks were obtained with CE. A protective antigen component was detected in the first protein peak (P-1) fraction, which was subsequently adsorbed onto DEAE-cellulose followed by elution by a linear gradient of NaCl. Two protein peaks were obtained. They uniformly contained an identical antigen, therefore were pooled (P-1'). P-1' was absorbed with anti-whole turkey serum antibodies by the use of an immunoabsorbent column to remove residual serum component originating from the growth medium. One protein peak (P-1") was obtained. In the gel diffusion and immunoelectrophoresis analyses P-1" produced one precipitin line against anti-CE serum. In sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) P-1" showed three major
protein bands corresponding to the molecular weights 43,000, 31,000 and 24,500, and one carbohydrate band which did not correspond to any of the three protein bands. Fifty micrograms of the P-1" antigens protected turkeys against challenge exposure to the homologous strain.
SEPARATION OF PROTECTIVE ANTIGENS IN A SALINE EXTRACT
OF PASTEURELLA MULTOCIDA TYPE 4

by

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Typed by Susan Schamp for Mohammed R. Bakhiet
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Fowl cholera is an acute septicemic disease of fowl caused by *Pasteurella multocida*. The disease has world-wide prevalence, and its high mortality devastates domestic poultry as well as wild fowl populations. Because of acuteness of the disease, treatment has little value for the control of the disease. Prevention is the only answer to the problem.

Two types of vaccine, bacterins (inactivated whole bacteria) and live vaccine, are currently available for prophylaxis. Occasional outbreaks do occur in chickens and turkeys which have been properly vaccinated by bacterins. A disadvantage of the live vaccine is a relatively high mortality observed after administration when the birds are under undefined stress factors (Lamont and Wichmann, 1977).

A saline extract of type 3 organism of *Pasteurella multocida* was found to be protective against fowl cholera in turkeys (Kodama et al., 1981). Soluble protective antigens were subsequently separated from the saline extract of type 3 and 1 organism of *Pasteurella multocida* by chromatographic methods by Syuto and Matsumoto (1982) and Kajikawa and Matsumoto (1984), respectively. The objective of the present study was to separate a protective antigen(s) from a type 4 strain of *Pasteurella multocida* and to study its (their) characteristics.
LITERATURE REVIEW

A. Fowl Cholera and its Etiology

Fowl cholera (avian cholera, avian pasteurellosis, avian hemorrhagic septicemia) is an infectious disease affecting domestic and feral birds. It is caused by Pasteurella multocida. It commonly appears as a septicemic disease with high mortality and morbidity, but chronic conditions sometimes occur. Hall et al. (1955) observed that in the chronic form of fowl cholera mortality could be low but infection persisted for at least four years. This disease is of historical importance because of its role in the early development of bacteriology, and because it was one of the four diseases the Veterinary Division of the USDA was created to investigate. Several outbreaks among fowl occurred in Europe during the latter half of the 18th century. The disease was studied in France by Chabert in 1782. Benjamin in 1851 gave a good description of the disease and demonstrated that it could be spread by cohabitation and formulated procedures for its prevention. In 1877 and 1878 Perroncito of Italy and Semmer of Russia observed in the tissues of affected birds a bacterium that had rounded form and occurred singly or in pairs. Toussant in 1879 isolated the bacterium and proved it was the sole cause of the disease (History according to Gray, 1913). A good description of the signs of the disease was reported as early as 1867 in Iowa where losses of chickens, turkeys and geese had occurred (USDA Monthly Report, 1867).

Pasteurella multocida is the causative agent of fowl cholera.
It is a Gram-negative, nonmotile, nonsporeforming rod occurring singly, in pairs and occasionally as chains or filaments. A capsule can be demonstrated in recently isolated cultures using indirect methods of staining. In tissues, blood and recently isolated cultures the organism stains bipolar.

*Pasteurella multocida* grows aerobically or anaerobically. The optimal growth temperature is 37°C. The optimal pH range is 7.2 - 7.8 depending on the growth medium. The bacterium grows on a simple medium such as tryptose agar but growth is enhanced when the medium is enriched with enzymatic digest of peptone, casein hydrolysate or avian serum but growth in such media alters colony morphology rapidly.

On primary isolation from birds with fowl cholera, colonies may be strongly or weakly iridescent, sectored with variable iridescence or blue with little or no iridescence. The composition of the medium determines to some extent the degree and type of iridescence. Occasionally an isolate produces blue colonies; when serum is added to the medium sectored or iridescent colonies are produced. Examination of 18-24 hour colonies with stereomicroscope using obliquely transmitted light is helpful when observing colonial morphology (Henry, 1933). Iridescent type is associated with outbreaks of acute fowl cholera and is highly virulent, while blue type is low in virulence and occurs commonly in flocks in which fowl cholera is enzootic (Hughes, 1930).

Heddleston et al. (1964) reported that a virulent isolate of *Pasteurella multocida* of avian origin produced iridescent colonies that dissociated *in vitro* and produced blue colonies. Gray (mucoid) colonies have not been reported in primary cultures from birds.
**Pasteurella multocida** is positive for indole reaction with some exceptions, catalase-positive, weakly positive for oxidase, and ferments glucose and sucrose. It is negative for hemolysis, nonmotile, does not grow in MacConkey's agar, does not ferment lactose, does not liquify gelatin and does not split urea.

Domestic fowl, game birds raised in captivity, and small feral birds that visit poultry yards are susceptible to fowl cholera. Most reported outbreaks involved chickens, geese, turkeys, and ducks. The disease usually occurs in young mature turkeys but all ages are highly susceptible. In addition to death losses, there is a decrease in egg production and often persistent localized infection in many birds.

How fowl cholera is introduced into a flock is often impossible to determine. There is no limit to the duration of the chronic carrier in fowl cholera other than the life of the bird. The birds usually harbor the organisms in the nasal clefts (Pritchett and Hughes, 1932), thus the survivors of an outbreak of fowl cholera may be the reservoir of infection. Wild birds, raccoons, rats, and mice which visit infected poultry yards and contaminated equipment used previously in infected poultry yards may introduce the disease into a flock. Dissemination of **Pasteurella multocida** within a flock is primarily by excretions from the mouth, nose, and conjunctiva of diseased birds that contaminate their environment, particularly the feed and water.

A presumptive diagnosis of fowl cholera may be made from clinical observations or necropsy findings. A common and convenient method of
presumptive diagnosis can be made by observation of bipolar-stained bacteria in a Giemsa-stained blood or liver smear. A conclusive diagnosis should be based on the isolation of the organism. Pasteurella multocida can be isolated from the viscera, bone marrow, heart blood, liver, meninges or localized lesions. It is less likely to be isolated from dehydrated, emaciated survivors of an acute outbreak. If birds are living, squeeze mucus from the nostrils or insert a cotton swab into the nasal cleft. Transfer the specimen to peptone broth and streak on a suitable medium.

Prevention of fowl cholera can be affected by eliminating the reservoirs of Pasteurella multocida. Good management practices, with emphasis on sanitation are the best means of preventing fowl cholera. Unlike many bacterial diseases, fowl cholera is not a disease of the hatchery. Infection therefore occurs after the birds are in the hands of the growers, and considerations must be given to the many ways that infection might be introduced into a flock. The primary source of infection is usually sick birds or those that have recovered and still carry the causative organism. Only young birds should be introduced as a new stock.

Vaccination should be considered in areas where fowl cholera is prevalent, but it should not be substituted for good sanitary practices.

B. Clinical Signs and Lesions

Pathogenicity of Pasteurella multocida in relation to fowl cholera is complex and quite variable, depending on the strain of Pasteurella multocida, the host species, and condition of contact between the two.
Heddleston et al. (1964) reported that the ability of *Pasteurella multocida* to invade and reproduce in the host is related to a capsule. Loss of ability of a virulent strain to produce the capsule results in the loss of virulence. However, some isolates from cases of fowl cholera have a substantial amount of capsule but are of low virulence. Therefore, the relationship between the capsule and virulence needs to be studied further.

Although it is convenient for descriptive purposes to refer to either acute or chronic fowl cholera, it is sometimes difficult to categorize the disease in this manner. The signs of infection and lesions that may occur may be intermediate to those described for acute and chronic forms of the disease.

The signs of acute infection are often present for only a few hours before death. Unless infected birds are observed during this period, death may be the first indication of the disease. Signs that often occur are fever, anorexia, ruffled feathers, mucus discharge from the mouth, diarrhea and increased respiratory rate. Cyanosis often occurs immediately prior to death. Sometimes affected birds show neurological signs. Fecal material associated with the diarrhea is initially watery and whitish in color, but later becomes greenish and contains mucus. Birds that survive the initial acute septicemic stage may later succumb to the debilitating effects of emaciation and dehydration, may become chronically infected but seldom recover.

Chronic fowl cholera may follow an acute stage of the disease or result from infection with organisms of low virulence. The signs are
generally related to localized infections. Wattles, sinuses, leg or
wing joints, foot pads and sternal bursae often become swollen.
Pharyngeal lesions and torticollis may be observed. Tracheal rales and
dyspnea may result from respiratory tract infection.

The lesions of acute fowl cholera are associated with vascular
disturbances. General hyperemia usually occurs. Hemorrhages are common
in lung, abdominal fat, subepicardial, peritoneal walls and subserosal
tissues. The liver may be swollen and usually contains multiple small
focal areas. Mature follicles often appear flaccid.

The lesion of the chronic infection are localized infections which
generally become suppurative and may be widely distributed anatomically.
They often occur in the respiratory tract and may involve any part
including sinuses and pneumatic bones.

C. Surface Antigens of *Pasteurella multocida* and Serology

Various serological tests were used to determine surface antigens
of *Pasteurella multocida* isolates. Carter (1955) employed indirect
hemagglutination test using antigen which was extracted with 0.85% NaCl
at 56°C for thirty minutes. Then the antigen was adsorbed to human type
0 erythrocytes. Clumping of the treated erythrocytes occurred in the
presence of homologous antisera. He found four types, A through D, by
this method. Nonencapsulated cultures were not typable by this test
which also failed to type mucoid strains, because a large amount of the
mucoid substance on the surface blocks the reaction. This blocking can
be prevented with many isolates if the hyaluronic acid in the extract is
depolymerized with hyaluronidase. Antigenic substances responsible for this reaction have not been biochemically characterized.

Namioka and Murata (1961) employed tube agglutination test to study surface antigens of isolates of Pasteurella multocida using HCl-treated whole bacterial cells and antiserum raised against them. The HCl-treated antigen was prepared by suspending sixteen hour culture in 1 N HCl then incubated in a plugged tube at 37°C for 16-18 hours, centrifuged and the sedimented organisms were washed. They found that the HCl-treated antigens of Pasteurella multocida were divided into two antigens: common and specific. However, Brogden and Packer (1979) reported that the tube agglutination tests were subject to non-specific agglutination; numerous cross-reactions were present even after the antiserum were adsorbed; in a few cases the homologous reaction was completely removed; the HCl-treated antigen from some cultures settled out after 24 hours in the typing antisera, in the normal serum, or saline control. They could not determine whether the cross-reactions were the result of a specific serologic reaction or the non-specific settling of the antigen. Namioka and Murata claimed that the HCl-treated antigen was the O antigen although they did not characterize the antigen.

Heddleston et al. (1972) used heat-stable formalized saline extracted antigen and antiserum raised against it in gel diffusion test to group Pasteurella multocida associated with fowl cholera. The antigen was prepared by suspending 18-24 hour culture in 0.85% NaCl solution containing 0.3% formaldehyde. The suspension was heated in a water bath at 100°C for one hour. The antigen appeared to be closely related but
not identical to the lipopolysaccharide-protein complex (endotoxin). Although the antibodies produced against such antigens generally detected the type-specific antigens, cross-reaction occurred between some types.

Brogden and Packer (1979) compared the various serological tests. They concluded that serotypes determined by one typing system generally did not correlate with serotypes determined by another system. Cultures of single serotype in one system often represented more than one serotype in another system. Because of the antigenic complexity of Pasteurella multocida and the nature of the antigens involved in each test, a reliable correlation between serotypes determined by different typing systems has not been made.

D. Immunity

There have been numerous attempts to produce efficient vaccines against fowl cholera (Carter, 1950; Heddleston and Hall, 1958; Heddleston, 1962), but results have not been consistent.

Langpap and Matischek (1970) have determined that chickens and turkeys may be infected with three immunologically distinct types of Pasteurella multocida and have noted that these three types are exemplified by strain X-73 (type 1), P-1059 (type 3) and P-1662 (type 4).

Two types of vaccines are currently available for prophylaxis. Bacterins, inactivated whole bacterial cells with an adjuvant, are injected systemically. Immunity induced by these bacterins is typespecific (Heddleston, 1962). Occasional outbreaks do occur in turkeys or chickens which have been properly vaccinated. These outbreaks are not caused by a difference in the antigenic structures between vaccinal
strain and field strains of Pasteurella multocida, because up to date evidence does not exist to support the possibility that an antigenic difference other than type specificity defined by Heddleston et al. (1972), constituted a marked difference in immunogenicity.

The second type of vaccine is a strain of Pasteurella multocida of low virulence which is given to turkeys in drinking water (Bierer and Derieux, 1972). It is more widely used than is the bacterin, particularly in meat producing birds in endemic areas. It does not only induce more reliable immunity than does a bacterin, but the immunity is also cross-reactive for many of the commonly occurring types of Pasteurella multocida. A disadvantage of this vaccine is a relatively high mortality observed after administration when the birds are under undefined stress factors (Lamont and Wichmann, 1977).

Not much is known about the identity of subcellular materials of Pasteurella multocida which are immunogenic. Subcellular component of Pasteurella multocida have been prepared by various methods, and their immunogenicity have been tested against fowl cholera. Rebers and Heddleston (1974) purified lipopolysaccharide–protein complex from Pasteurella multocida. Their study on its physical and chemical composition indicated that it contained endotoxin. It was immunogenic and toxic in chickens. It was also cross-reactive with some of the other serotypes.

Phillips et al. (1981) showed that in contrast to a report on the effectiveness of the ribosomes as protective immunogens (Baba, 1977), ribosomes from nonencapsulated Pasteurella multocida were not protective in mice challenge exposed with encapsulated cells, thus supporting
the view that contamination with other cell components in the ribosomal vaccine is the actual moiety of the vaccine.

A protective antigen was also found in a soluble fraction prepared by extracting *Pasteurella multocida* with potassium thiocyanate (Gaunt et al., 1977), or with sodium salicylate (Gilmour et al., 1979), but these antigens have not been characterized.

Carter (1955) found that the serological specificities of a saline extracted antigen was correlated with protective specificities. It was inferred that the saline extracted antigen was protective. Kodama et al. (1981) found that a crude saline extracted antigen provided 80% to 100% protection in young adult turkeys against fowl cholera. The crude saline extracted antigen contained approximately 5% of endotoxin. Minor contamination of the immunizing preparation by endotoxin may elicit the formation of anti-endotoxic antibodies (Strim et al., 1967). Also it is known that endotoxins from some types are cross-reactive (Rebers and Heddleston, 1974). In order to confirm that the saline extracted antigen is immunogenic it is essential that it should be separated in a form that does not react with antisera prepared against heterologous strains. Syuto and Matsumoto (1982) separated and characterized type 3 (P-1059) organism of *Pasteurella multocida*. In 1984, Kajikawa and Matsumoto separated and characterized type 1 (X-73) and found that the chromatographically separated antigens (CSA) of type 1 and 3 of *Pasteurella multocida* were antigenically distinct in a gel diffusion analysis.
**MATERIALS AND METHODS**

**Bacterial strain** - *Pasteurella multocida*, strain P-1662 belonging to Heddleston's type 4 (Heddleston et al., 1972), was supplied by Dr. K. R. Rhodes, National Animal Disease Center, Ames, Iowa. After 4 passages in turkeys, the organism from a single colony was propagated on sheep blood agar, harvested in brain-heart infusion broth (Difco Laboratories, Detroit, Mich.) and stored frozen at -70°C. The frozen culture was used to produce antigen and infect turkeys.

**Preparation of crude extract (CE)** - The frozen culture was recovered on blood agar. Ten colonies were picked and subcultured on blood agar. Then 8 hour growth on a single plate at 37°C was harvested in 100 ml brain heart infusion broth. The bacterial suspension in 0.5 ml containing approximately $10^8$ colony forming units was spread onto agar medium which was prepared as follows: Brain-heart infusion broth (Difco Laboratories, Detroit, Mich.) 26 gm; Casamino acids (Difco) 5 gm; hemin (Sigma chemical Co., St. Louis, MO) 10 gm; peptone (Daigo National Chemical Co., Osaka, Japan) 5 gm; and double distilled water, 940 ml were mixed. The pH was adjusted to 7.2. Bacto-agar (Difco), 12 gm were added and the medium was autoclaved at 120°C for 30 min. Then 50 ml of sterile heat inactivated turkey serum and 50 ml fresh yeast extract (Hayflick, 1965) were added aseptically. The agar medium was then plated in Petri dishes (150 x 15 mm). The inoculated plates were incubated at 37°C for 19 hours. The bacterial growth was harvested in a 2.5% NaCl solution (7 ml/plate) and agitated for 1 hour at 56°C as described by Maheswaran et al. (1973). The suspension was centrifuged
at 20,000 x g for 20 minutes and the supernatant was recentrifuged 2 more times, dialyzed for 72 hours in a 0.85% NaCl solution containing 0.02% NaN₃. This solution constituted CE.

**Antiserum** - Adult New Zealand White rabbits originating from a Pasteurella-free colony (Rabbit Research Center, Oregon State University) were used. Monthly nasal cultures of the rabbits were consistently negative for *P. multocida*, and their preimmune sera were negative for antibodies against the CE in a gel diffusion test. The rabbits were subcutaneously injected with 0.5 ml of CE emulsified in an equal amount of Freund's complete adjuvant. The injection was repeated twice with 0.5 ml of CE emulsified in Freund's incomplete adjuvant at 30 day intervals. Fourteen days after the third injection the rabbits were exsanguinated. The antiserum against CE will be referred to as rabbit anti-CE serum.

**Gel filtration** - A 3 ml portion of CE was applied to a Sephadex G-200 superfine (Pharmacia, Piscataway, New Jersey) column (2.5 x 36) equilibrated with 0.01 M sodium phosphate buffer, pH 7.2. The material was eluted at 2 ml/hr. at 4°C and 3 ml fractions were collected.

**Ion-exchange chromatography** - The first peak (P-1) fractions of Sephadex G-200 effluent from several runs were pooled and concentrated. This fraction (P-1) was adsorbed onto a DEAE-cellulose column (1.5 x 30 cm) equilibrated with 0.01 M sodium phosphate buffer, pH 7.2. It was eluted by a linear gradient of NaCl from 0 to 1.0 M with a flow rate of 20 ml/hr. at 4°C.

**Immunoadsorbent column** - Whole turkey serum (25 ml) was coupled to 15 ml of Sepharose 4B (Pharmacia) by the method of Avrameas and
Ternynck (1967). Rabbit antiserum against whole turkey serum was passed through the absorbent column and antibodies were eluted with 3M KSCN at 4°C. The eluted antibodies were in turn coupled to 15 ml Sepharose 4B.

**Immunodiffusion test** - Agar gel diffusion tests were done with standard 6 well Ouchterlony pattern in 1% agarose.

**Immunoelectrophoresis** - Immunoelectrophoresis was done by the method of Scheidegger (1955) by electrophoresing samples at 6 V/cm in 1% agarose in 0.05M barbital buffer, pH 8.5.

**Electrophoresis** - Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was done according to Weber et al. (1972) using 8% acrylamide gel and the following protein standards: human IgG (Miles Laboratories, Elkhart, Indiana); bovine serum albumin (Sigma Chemical Co., St. Louis, Missouri); ovalbumin (Miles); myosin (Sigma); and cytochrome C (Sigma). Protein bands were stained with Coomassie brilliant blue R (Sigma). Carbohydrate band was stained with periodic acid-Schiff reagent.

**Protection test** - Test antigens in 0.5 ml amounts emulsified in an equal amount of Freund's incomplete adjuvant were injected twice subcutaneously at two week intervals into 11 week-old Bronze turkeys in the first test. Two weeks after the second injection the birds were intramuscularly inoculated with 200 colony forming units (CFU) of *Pasteurella multocida* strain P-1662. Mortality was checked daily for 2 weeks. Survivors were killed and liver swab cultures were taken to isolate *Pasteurella multocida* (Kodama et al., 1981). For the second test 21 week-old Nicholas Large White turkeys were treated with the same
immunization schedule, and challenge-exposed with $2.2 \times 10^3$ CFU of P-1662 strain.

**Chemical analysis** - Protein concentration was determined by the method of Lowry et al. (1951) with bovine serum albumin as standard. Total carbohydrate content was determined by the phenol-sulfuric acid method with glucose as the standard (Dubois et al., 1956).
**RESULTS**

**Gel filtration** - The crude extract was fractionated on a Sephadex G-200 superfine column. Two protein peaks were obtained (Fig 1). Chemical analysis of protein and carbohydrate of the two peaks is shown in (Table 1). Fractions corresponding to the two protein peaks (P-1 and P-2) were pooled separately then concentrated. Gel diffusion tests revealed that the two protein peaks contained different antigens. The first protein peak contained a single antigen while the second protein peak contained at least another antigen unrelated to the first protein peak antigen (Fig 2). In immunoelectrophoresis assay the first protein peak produced one precipitin line against rabbit anti CE serum containing 1:200 dilution of normal turkey serum (Fig 3), while the second protein peak produced two precipitin lines (Fig 4); neither corresponded to the P-1 precipitin line. The P-1, P-2 and CE in a dose of 100, 100 and 200 μg protein respectively, were emulsified in the Freund's incomplete adjuvant and injected subcutaneously into turkeys. The turkeys were challenge-exposed to live P-1662 strain 14 days after the second vaccination (Table 2). All the birds which received P-1 fraction were protected whereas 89%, 33% and 0% were protected for the CE, P-2 and control groups respectively. *Pasteurella multocida* was isolated from 22%, 0%, 78% and 100% for CE, P-1, P-2 and control groups respectively. The statistical analysis showed highly significant difference (P < 0.01) between P-1 and P-2 fractions. Analysis by SDS-PAGE revealed that P-1 contained 4 major protein components (Fig. 5). None of the protein
Fig 1. An elution profile of gel filtration with a Sephadex G-200 superfine column (2.5 x 36 cm) of CE of Pasteurella multocida type 4. A 3 ml portion was applied to the column and eluted at a flow rate of 2 ml/hour in 0.01 M phosphate buffer (pH 7.2). The protein concentration ( ) was determined by the Lowry's method and the carbohydrate content was determined by the phenol-sulphuric acid method ( ).
Table 1. Protein and carbohydrate contents of various fractions obtained from Pasteurella multocida strain P-1662.

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<th>Antigen</th>
<th><strong>Carbohydrate</strong>&lt;sup&gt;a&lt;/sup&gt;</th>
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<td></td>
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<td>recovery</td>
<td>µg/ml&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Crude extract</td>
<td>1100</td>
<td>100</td>
<td>1640</td>
</tr>
<tr>
<td>Gel filtration</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P-1</td>
<td>251</td>
<td>23</td>
<td>182</td>
</tr>
<tr>
<td>P-2</td>
<td>198</td>
<td>18</td>
<td>536</td>
</tr>
<tr>
<td>Anion exchange chromatography</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P-1'</td>
<td>118</td>
<td>11</td>
<td>77</td>
</tr>
<tr>
<td>Absorption with anti-turkey serum</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P-1''</td>
<td>80</td>
<td>7</td>
<td>51</td>
</tr>
</tbody>
</table>

<sup>a</sup> Carbohydrate concentrations were estimated by the phenol-sulfuric acid method with glucose as the standard.

<sup>b</sup> Protein concentrations were estimated by the method of Lowry et al. (1951) with bovine serum albumin as the standard.

<sup>c</sup> The volume of each fraction was adjusted to that of the original crude extract.

<sup>d</sup> Carbohydrate/protein.
Fig 2. Comparison of the P-1 and P-2 antigens in a gel diffusion test. Wells 1 and 4 contained CE, 2 and 6 contained P-1 and 3 and 5 contained P-2 and normal turkey serum respectively. In (A) the center well contained rabbit anti CE serum while in (B) it contained rabbit anti CE serum mixed with a 1:50 dilution of normal turkey serum.
Fig 3. Comparison of the precipitin lines produced by P-1 and CE in immunoelectrophoresis assay. Wells 1 and 2 contained CE and P-1, respectively. The central trough contained rabbit anti CE serum mixed with a 1:200 dilution of normal turkey serum.
Fig 4. Comparison of the precipitin lines produced by P-2 and CE. The upper and lower well contained P-2 fraction and CE, respectively. The central trough contained rabbit anti CE serum mixed with a 1:50 dilution of normal turkey serum.
Table 2. Immunogenicity of CE, P-1 and P-2 fractions obtained from Pasteurella multocida strain P-1662a.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Protein (µg/dose)</th>
<th>Mortality (No. of dead/total)</th>
<th>Median death time (days)</th>
<th>Pasteurella multocida isolation (No. of pos./total)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>200</td>
<td>1/9</td>
<td>14</td>
<td>2/9</td>
</tr>
<tr>
<td>P-1</td>
<td>100</td>
<td>0/9</td>
<td>-</td>
<td>0/9</td>
</tr>
<tr>
<td>P-2</td>
<td>100</td>
<td>6/9</td>
<td>11.2</td>
<td>7/9</td>
</tr>
<tr>
<td>Unvaccinated</td>
<td>-</td>
<td>11/11</td>
<td>1.5</td>
<td>11/11</td>
</tr>
</tbody>
</table>

*The birds were vaccinated twice with antigens in designated amounts emulsified in Freund's incomplete adjuvant with a 14 day interval. Fourteen days after the second vaccination they were inoculated intramuscularly with 200 CFU of the homologous strain.*
Fig 5. SDS-PAGE of CE, P-1 and P-2 extracted from Pasteurella multocida, strain P-1662. Lanes = a, markers; b, extract of growth medium; c, CE; d, P-1; e, P-2. The numbers on the left represent the molecular weights of the markers.
bands corresponded to those found in the growth medium. The P-2 contained protein bands which corresponded to those found in the growth medium.

**Ion exchange chromatography** - The P-1 fraction was adsorbed onto a DEAE-cellulose column, and eluted by a linear gradient of NaCl. It produced protein peaks (Fig 6). Fractions corresponding to each protein peak were pooled separately then concentrated. In a gel diffusion test the two protein peaks produced identical precipitin lines against rabbit anti CE serum (Fig. 7). Therefore the two protein peaks were pooled (P-1'). The chemical analysis of protein and carbohydrate of P-1' is shown in (Table 1). In a gel diffusion test P-1' produced 1 precipitin line against rabbit anti CE serum containing 1:200 dilution of normal turkey serum (Fig 8). In immunoelectrophoresis assay P-1' produced 1 precipitin line against rabbit anti CE serum containing 1:200 dilution of normal turkey serum (Fig 9).

**Removal of residual turkey serum** - The pooled fraction (P-1') from the anion exchange chromatography was passed through the immunoadsorbent column to remove any residual turkey serum originated from the growth medium. It produced 1 protein peak (P-1''). In a gel diffusion test P-1'' produced 1 precipitin line against rabbit anti CE serum containing 1:200 dilution of normal turkey serum (Fig 8). In immunoelectrophoresis assay P-1'' produced 1 precipitin line against either rabbit anti CE serum or rabbit anti CE serum containing 1:200 dilution of normal turkey serum (Fig 10). Analysis by SDS-PAGE revealed that the P-1'' contained 3 major protein components with molecular weight of approximately 43,000, 31,000 and 24,500 (Fig 11 and 12). Molar ratio of the
Fig 6. DEAE-cellulose chromatography of P-1 fraction. The fractions obtained from several gel filtration runs were pooled, applied to a DEAE-cellulose column (1.5 x 30 cm) and eluted by a linear gradient of NaCl sodium phosphate buffer, pH 7.2. Protein concentrations (µg/ml) were determined by the method of Lowry (1951).
Fig 7. Comparison of the precipitin lines produced by P-1, D-1 and D-2. Wells 1, 3 and 5 contained CE, while wells 2, 4 and 6 contained P-1, D-1 and D-2 respectively. In (c) D-1 and D-2 had been passed through the immunoadsorbent column. The central well in (a) and (c) contained rabbit anti CE serum while (b) contained rabbit anti CE mixed with a 1:50 dilution of normal turkey serum.
Fig 8. Comparison of P-1, P-1', P-1" and CE antigen in a gel diffusion test. Wells 2, 4 and 6 contained P-1, P-1' and P-1", respectively. Wells 1, 3 and 5 contained CE. The central well contained rabbit anti CE serum mixed with a 1:200 dilution of normal turkey serum.
Fig 9. Comparison of the precipitin lines produced P-1 and CE in immunoelectrophoresis assay. Wells 1 and 2 contained CE and P-1' respectively. The central trough contained rabbit anti CE serum mixed with a 1:200 dilution of normal turkey serum.
Fig 10. Comparison of the precipitin lines produced CE and P-1". Wells 1 and 2 contained CE and P-1" respectively. The central trough (a) contained rabbit anti CE serum, while the central trough (b) contained rabbit anti CE mixed with a 1:200 dilution of normal turkey serum.
Fig 11. SDS-PAGE of the P-1". Lanes: a, markers; b, P-1" stained for proteins with Coomassie blue; c, P-1" stained for carbohydrate with periodic acid-Schiff reagent. The numbers on the left represent the molecular weights of the markers.
Fig 12. Molecular weights of the three protein components of the P-1", determined by SDS-PAGE.
three protein components 43,000:31,000:24,500 was 22:9:33. The antigen also contained one carbohydrate component which did not correspond to any of the protein bands (Fig 11). The P-1" of this type (type 4) did not react with rabbit anti CE serum of type 1 and 3 (Fig 13). Chemical analysis of protein and carbohydrate of the P-1" is shown in (Table 1). The carbohydrate/protein ratio was 1.56. The P-1" was eluted in a 38% of the total column volume of Sepharose 6B in a 0.85 NaCl solution when its void volume was 35% indicating that the antigen had a molecular weight of approximately $4 \times 10^6$.

Protection test - Immunogenicity of CE, P-1, P-1' and P-1" were tested in turkeys. Protein amounts per dose were 328, 100, 50 and 50 for CE, P-1, P-1' and P-1" respectively. After the second vaccination the birds were challenge-exposed to the homologous strain. All the vaccinated birds were protected while all the control unvaccinated birds died and *Pasteurella multocida* was isolated from all of them (Table 3).
Fig 13. Comparison of the chromatographically separated antigens (CSA) of type 1, 3 and 4 of Pasteurella multocida in the gel diffusion test. Wells 1, 2 and 3 contained the CSA of type 1, 3 and 4. The center wells in a, b and c contained rabbit anti CE serum against type 1, 3 and 4 respectively.
Table 3. Immunogenicity of various antigens obtained from *Pasteurella multocida* strain P-1662.\(^a\)

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Protein (ug/dose)</th>
<th>Mortality (No. of dead/total)</th>
<th>Pasteurella multocida isolation (No. of pos./total)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>328</td>
<td>0/10</td>
<td>0/10</td>
</tr>
<tr>
<td>P-1</td>
<td>100</td>
<td>0/9</td>
<td>0/9</td>
</tr>
<tr>
<td>P-1'</td>
<td>50</td>
<td>0/10</td>
<td>0/10</td>
</tr>
<tr>
<td>P-1''</td>
<td>50</td>
<td>0/10</td>
<td>0/10</td>
</tr>
<tr>
<td>Unimmunized control</td>
<td>---</td>
<td>12/12</td>
<td>12/12</td>
</tr>
</tbody>
</table>

\(^a\)The birds were vaccinated twice with antigens in designated amounts emulsified in Freund's incomplete adjuvant with a 14 day interval. Fourteen days after the second injection they were inoculated intramuscularly with 2200 CFU of the homologous strain.
DISCUSSION

The origin of the saline extracted antigen is not known. Antigens which were extracted by procedures similar to one employed in this study had been referred to as capsular antigens (Kodama et al., 1981), but no effort was made to determine the exact topographical location of this antigen. The CE contained at least 3 antigenic components (Fig 3). The fraction P-1 contained 1 antigenic component (Fig 3), while the P-2 fraction contained 2 antigenic components (Fig 4). The fraction P-1, but not P-2 contained a protective antigen (Table 2). This protective antigenic component was adsorbed onto DEAE-cellulose column and then passed through immunoadsorbent column to produce the P-1". The P-1" contained 3 protein components with molecular weight of approximately 43,000, 31,000 and 24,500 and 1 carbohydrate component (Fig 11). Syuto and Matsumoto (1982); and Kajikawa and Matsumoto (1984) showed that the chromatographically separated antigens (CSA) of Pasteurella multocida type 1 or 3 contained 1 carbohydrate component and a few protein components. The P-1" of Pasteurella multocida, type 4, did not react with rabbit anti-serum made against the type 1 and 3 CE. This indicated that the P-1" was type-specific (Fig 13).

The two antigenic components in P-2 cross-reacted with type 1 and 3 (unpublished data). These 2 antigenic components were not protective in turkeys (Table 2).
The protective antigens prepared by this procedure could have higher immunogenicity than the bacterins since the protective antigens would be the only components of the vaccine, thus eliminating other possible interfering bacterial substances. However, this type of vaccine induces type-specific immunity (Fig 13). Antigens derived from several prevalent types may have to be mixed for the proper protection.

The P-1" antigens could have advantage over heat-stable formalized saline extracted antigen in the gel diffusion test (Heddleston et al., 1972) since it does not cross-react. In fact a sensitive enzyme-linked immunosorbent assay has been developed with type 3 CSA and found very useful for assaying levels of protective immunity (Matsumoto et al., unpublished data).

The present study demonstrated that the crude extract (CE) consisted of at least 3 components (Fig 3). Only one component was immunogenic while the other two were not (Table 2). The immunogenic component (P-1) can be separated by gel filtration which also separated all components of the growth medium (Fig 5) except a residual amount of turkey serum which was removed by absorption with anti-whole turkey serum to produce P-1". P-1" contained antigenically similar components as indicated by the gel diffusion test (Fig 8) or immunoelectrophoresis assay (Fig 10). The approximate molecular weight of the P-1" was 4 \times 10^6. The P-1" contained at least 3 protein components of molecular weight 43,000, 31,000 and 24,500. Molar ratio of the three protein components (43,000:31,000:24,500) was 22:9:33. It also contained one carbohydrate component which did not correspond to any of the 3 protein
components (Fig 11). The carbohydrate/protein ratio was 1.56 (Table 1). The P-1" in a dose of 50 μg protein protected adult turkeys against challenge exposure with the homologous strain (Table 3). The results obtained from gel diffusion test (Fig 12) suggested that the P-1" was type-specific. However, careful cross-protection studies are needed to confirm the type specificity of CSA. P-1" antigens could have advantage over stable formalized saline extracted antigen in gel diffusion test (Hedlestone et al., 1972) since it does not cross-react (Fig 13).
REFERENCES


