CHAPTER 1

INTRODUCTION

Pasteurella multocida have been isolated from a wide variety of mammals and birds and have been named accordingly. Although there were demonstrable differences between these strains from different sources, the essential characteristics were so similar that all were included under the multocida or septica species. They were: (1) *P. bollingeri* or *boviseptica*, (2) *P. avicida* or *aviseptica*, (3) *P. cuniculicida* or *lepiseptica*, (4) *Bacterium bovicida* or *P. bubalseptica*, (5) *P. vituliseptica*, (6) *P. muricida* or *Bacillus bipolaris*, (7) *P. pneumotropica* and (8) *P. gallinarum* [1,2]

Effective immunizing agents are in demand in many parts of Thailand and elsewhere for the prevention of Pasteurella infections which are economically important diseases such as shipping fever, fowl cholera and hemorrhagic septicemia. Acute infection of these diseases can cause rapid death of the individual and rapid spread of the disease through a herd, making difficulty for effective antimicrobial therapy. The only way to control this disease is by efficient vaccination.

At the present, vaccine used in prevention of fowl cholera in Thailand is formalinized - killed whole cell vaccine prepared from Department of Live stock.

For improvement of appropriate vaccination, the main objective of this research project is the protective study of immunoglobulins prepared from whole cell and capsular antigens of Pasteurella multocida.

1. Description of Pasteurella multocida

1.1 Taxonomy

P. multocida was classified according to Bergey [3] as:

Genus	Pasteurella
Tribe	Pasteurelleae
Family	Brucellaceae
Order	Eubacteriales
Class	Schizomycetes

1.2 Microscopic morphology and growth characteristics

P. multocida was usually coccobacillary or short rod (0.25 micron (μ) to 0.4 μ by 0.6 μ to 2.6 μ in size) in culture from diseased tissue; strain from healthy animal was often pleomorphic with longer bacillary form and occasional short filament. It was generally capsulated, but noncapsulated forms have been reported. It was gram - negative, bipolar staining, nonmotile and nonspore - forming. [3,4]

The organism was an aerobe and facultative anaerobe. Its optimum temperature for growth was 37° C. The pH growth range was from 6 to 8.5 with an optimum of 7.2 to 7.4 [4]

The use of digested protein media or proteose peptone stimulated the growth of the organism. The organism could be grown in beef infusion media, but better growth was obtained from blood or chocolate agar where it produced small, nonhemolytic, translucent colonies with a characteristic

musty odor. (4,5,6) Non - hemolytic, but most strains produced a brownish discoloration of blood media in region of confluent growth. (3) It required nicotinamide and pantothenic acid as accessory growth factors. (1)

The organism had three main colonial types: S (smooth), M (mucoid), and R (rough). The smooth type was also called the fluorescent type. To this type belong almost all freshly isolated cultures which possessed well - developed capsules. A culture of the mucoid type possessed the mucoid antigen with or without the capsular antigen. In cultures of the rough type, also called blue type, no capsular nor mucoid antigen had been demonstrated. (7)

Bain (1954) divided the colonial forms of P. multocida into several phases, I and others. It was considered that phase I corresponds to the culture of Carter's type B which possessed an unknown masked antigen other than the known capsule or, in other words, that the culture of phase I was the fully immunogenic form of type B. He suggested that the phase may be a mucoprotein surface antigen. (7,8)

Colonial forms could transform from virulent to avirulent strains or in the sequence mucoid to smooth to rough. (1) The dissociation pattern of colonial variants was as in table 1 page 4 (1,9)

Table 1 Classification of Colonial Variants of Pasteurella multocida

Older Designations	Proposed Designations		Capsule	Reaction in
	Colonial	Antigenic		Acriflavine
Mucoid.....	mucoid	M	+ +	slimy precipitate
Fluorescent.....	smooth	S	+	cells remain in suspension
Intermediate.....	smooth	S	+	partial floccula- - tion
Granular Blue.....	smooth	SR	-	flocculation
Rough.....	rough	R	-	flocculation

1.3 Biochemical characteristics

Their fermentation of several carbohydrates without forming gas was characteristic. (1) Rosenbusch and Merchant (1939) divided P. multocida strains into three groups on the basis of sugar fermentations as shown in table 2 page 4 (5,9,10)

Table 2 Types of Pasteurella

Sugar	Pasteurella multocida			Pasteurella
	Type 1	Type 2	Type 3	haemolytica
Lactose	-	-	-	+
Xylose	-	+	+	+
Arabinose	+	-	+	±
Dulcitol	+	-	+	±

1.4 Pathogenecity

P. multocida was pathogenic for a large number of different animals; however, the organism varied in virulence from the highly virulent to the comparatively avirulent types. Probably the most important role that P. multocida played in disease was that of a secondary invader when the resistance of the animal had been reduced by various stresses. (4)

The more susceptible of the experimental animals were the mouse, rabbit, and pig. Guinea pigs were more resistant. Chickens were more susceptible to fowl strains, although highly virulent ovine and bovine strains were able to produce death. (4,9)

1.4.1 Antigenic structure

The antigens of P. multocida were many and complex. Many attempts had been made to identify serological varieties of P. multocida.

Rosenbusch and Merchant (1939) classified Pasteurella into four groups by means of agglutination tests and concluded that there was no evidence to justify a zoological classification on the basis of serological properties or biological characteristics. (7,10)

By means of slide agglutination tests Little and Lyon (1943) identified three serotypes which were referred to as type 1,2 and 3; each type having no correlation with host specificity. (11,12,13)

Robert (1947) divided his strains into four principal types, 1 to 4 or groups by means of serum - protection tests in mice. (4,14)

Carter (1955) employing an indirect hemagglutination test, was able to identify four different types on the basis of difference in capsular substances. (K antigens) (group A,B,C,D) (7,8,15)

It was tentative concluded that the protection substance of Pasteurella organism obtained by Robert (1947) was the same as the K antigens.

Carter (1955) demonstrated a strong correlation between serological types and host species: type B strains included most of those causing epizootic pasteurellosis or hemorrhagic septicemia of cattle [16]; type A strains were isolated frequently from cases of fowl cholera [15]; type C cultures occurred infrequently, and at first they had been believed to come from dogs and cats, but this was not confirmed and the category was subsequently abandoned [17,18]; type D strains were associated with a wide range of infections in cattle, sheep, pig and poultry, including for example, shipping fever of cattle, viral pneumonia of pig, sporadic pneumonia in cattle and pig, fowl cholera, and bovine matitis. Strains of P. multocida recovered from human infections were of type A and D.[19]

Later, a further - type E - was recognized by Carter (1961) and type C was shown to be not true capsular type. There were therefore only four capsular types, namely A,B,D and E [20,21]

Carter's type A and B were probably the same as Roberts' 2 and 1, respectively; C and D might correspond to Roberts' 3 and 4.

To reveal the somatic antigen Namioka and Murata (1961) removed the capsular material by treatment with N HCl, suspended the washed organisms in saline at pH 7.0 and tested them against antisera prepared in rabbits by injection with similarly decapsulated organism. By the use of agglutination and absorption tests, they established six O group on the basis of somatic antigens [12]; and Ogata (1968) found that by a combination of capsular and somatic antigens 15 serotype could be recognized containing 12 O groups.[22]

The question arised as to what type of substance remained as the somatic antigen after the HCl treatment. Bain (1961)[7] considered that it consisted of a part of the cell wall substance and therefore, it was probably appropriate to cell such a somatic antigen a cell wall antigen. Therefore, it was assumed that the substance was a lipopoly-saccharide combined with a protein. [7]

According to the most widely used system, P. multocida was typed serologically by the indirect hemagglutination method of Carter (1955) which determined capsular type, and the direct agglutination test of Namioka and Murata (1961) which identified somatic antigens (O group) of acid extracted cell.

In their expanded classification, the identification of a serotype required the recognition of both the capsular and somatic components, the former being designated by a capital letter and the latter by an arabic number. Two important serotypes causing fowl cholera were designated 5 : A and 8 : A. [1,3,4,9]

The following table related Carter's classification with the earlier designations. [9]

Carter.....	Types	A	B	C	D
Rosenbusch and Merchant.....	Groups	1	2		
Little and Lyon.....	Types	1	2	3	
Roberts.....	Types	2	1	3	4

Carter (1957) gave further attention to colonial variation and antigenic characteristics of P. multocida. Colonial morphology was studied by obliquely transmitted light, and antigenic characteristics by behavior with 1 : 1000 acriflavine solution. His proposed designations were related to older designations as shown in table 1 page 4 [9,21]

Characteristics of the O antigen of P. multocida were examined with the following results: (7)

- Because there were many stages of loss variation of capsular antigen or mucoid variation in the organism, it was difficult to obtain constant O agglutination. O antigens of the organism could only be prepared by treating the culture with N HCl. With such antigens, true O antisera could be produced.

- It seemed that the O antigen of the organism did not change readily to rough antigen when cultures were subcultured for a year or more.

- In all the culture employed the organisms possessed O antigenic relationships.

- By absorption tests, O antigens of P. multocida were divided into two antigens, common and specific.

- Cultures belonging to types A and D of Carter (1955) could be divided into two types according to their specific O antigen (O group)

No exotoxins had been demonstrated but powerful endotoxins were present in some strains. (5) Weil had demonstrated nontoxic aggressins or " infection - favoring " substances in the inflammatory exudates of Pasteurella - infected animals. (4)

1.4.2 Diseases caused by Pasteurella multocida

P. multocida was the primary causative agent of fowl cholera and of hemorrhagic septicemia of rabbits, rats, horses, sheep, dogs, cats, chinchilla, and swine. It was also often found to be associated with pneumonia in various animals. (23,24)

Fowl cholera (avian pasteurellosis) was an infectious disease affecting practically all species of fowls. It usually appeared as a septicemic disease associated with high morbidity and mortality, but chronic manifestations were also of frequent occurrence. P. multocida infection was primarily a resiratory disease. The response when the organisms were introduced intranasally was varied, some chickens developing acute septicemic cholera and others developing localized infections such as rhinitis and wattle involvement. Still others became healthy carriers. In occurrence of outbreak, there must be some secondary factors such as season, weather, or nutrition. Inadequate ventilation had also been suggested as an important contributing factor to the spread and severity of fowl cholera outbreaks. The actual number of organisms in the air required to produce disease was less than the number required by subcutaneous injection. (9)

- Symptoms: The disease occured in all age group, except during the first month of life, when it was very seldom seen. Peracute, acute and chronic forms were known. In peracute cases death without previous symptoms might be the first indication of the disease. Symptoms in acute forms included anorexia, thirst, fever, ruffled feathers, mucous discharge from the mouth, diarrhea, first with mucoid white droppings followed by light greenish one. In chronic form swollen hock joints or emaciation often occured. (25)

- Gross lesions: In peracute cases a few petechial hemorrhages might be the only lesions observed. In acute form of the disease usually wide - spread hemorrhages occured in different serus and mucous membrane. The liver was usually enlarged, sometimes covered with a thin layer of

fibrin, that might be easily removed. Under the cap of the liver, many pinpoint to millet corn size yellow necrotic foci might be observed. These small necrotic foci were only visible if we look very carefully at the liver and washed the surface of the liver with water. Sometimes smaller and larger necrotic foci were present. In the abdominal cavity a small quantity of yellowish exudate might be present. The spleen was sometimes normal, or might be enlarged and contained small, yellow necrotic foci. (25)

Petechial or echymatic hemorrhages occured in the pericardium, and epicardium, especially on the coronary band. The heart of water fowl shew larger hemorrhages than poultry. Similar hemorrhages might occur in the visceral serosa in the air sacs, in syrinxes, and in the mucous membrane of the intestines. (25)

The serosa of the small intestines was hyperaemic, their mucous membrane was also markedly hyperaemic, swollen, and might contain hemorrhages. The annular bands of the small intestines were swollen, and might be white or reddish in color. The intestinal contents were usually waterly, mixed with large amounts of viscid mucus. (25)

The pathanatomical changes of fowl cholera were very similar to the changes found in duck plaque. The different diagnosis might be based on changes in oesophagus and cloaca while in fowl cholera oesophagus and cloaca were without changes, in duck plaque they used to have pathognomonic lesions. (25)

If the clinical symptoms and the changes did not give a clear basis for diagnosis, microscopic examination of the blood or livers smear, bacteria culture and virus isolation should be attempted. Many bipolarly

staining, small rods in a freshly dead ducks'blood or liver must cause suspicion of cholera, which should be ascertained by cultural examination.(25)

Because it had been rarely recognized as an infection of man the mechanism of infection was not well known. It might be introduced through bite wounds, or latent infection in the respiratory tract might be mobilized by injury or coexisting disease. (1)

Pasteurellosis in man followed two principal patterns: (1) a wound infection most commonly originating with an animal bite or scratch and (2) nontraumatic infections, often more or less obviously related to the respiratory tract but with no demonstrable extraneous source of the agent. In both cases, infections might reach other organ systems but were usually traceable to one of these two kinds of entry portals. Occasionally, cases did not readily fit into either of these types (e.g. urinary tract infection, CNS involvement, enteritis, and peritonitis) (26)

The organism was usually transmitted by oral or nasal excretions from infected animals and ordinarily entered tissues through mucous membranes of the upper air passage but might gain entrance through the conjunctiva or cutaneous wounds. (27)

Since many cases of human pasteurellosis, particularly those affecting the respiratory tract, could not be traced to animal contacts, the possibility of P. multocida leading a commensal existence in the human upper respiratory tract had been raised. (26)

1.5 Resistance

P. multocida was readily destroyed by 3 percent cresol, 1 percent phenol, 0.1 percent formalin, and 1 : 5000 bichloride of mercury. At 60 C,

the organism was destroyed in 10 minutes. It remained viable in manure for at least a month and about 3 months in the decaying carcass and in garden soil. Ducks dead of cholera should be burned or buried deep, because the cholera organism could survive for long periods in carcasses. The organism survived in dried blood smears on glass for 8 days at room temperature. (1,4,9,28,29)

Stock cultures died shortly after being stored in the refrigerator; however, most air - tight cultures could survive for months and even years when stored at room temperature. (4)

Virulent stock cultures could be preserved on blood agar in sealed tubes or by lyophilization. (1)

This organism was sensitive to many of the commonly used antibiotics such as penicillin, chloramphenicol, tetracycline, polymyxin B, erythromycin or carbomycin (1); however considerable resistance to streptomycin, bacitracin, neomycin, and viomycin was reported by Muysson and Carter (1959). The sulfa compounds, especially sulfathiazole, sulfamethazine, and sulfamerazine, were effective against it in vivo. (4,30) The organism was very susceptible to penicillin (2 - unit disc) in vitro. (6)

Because P. multocida was sensitive to as little as 0.1 unit of penicillin per milliliter of culture media, it could be differentiated from other gram negative bacilli, particularly Haemophilus influenzae, with which it was easily confused in routine bacteriologic diagnosis. (31)

1.6 Treatment, Prevention and Control

Sulfonamides, and antibiotics could be used in infected flocks to reduce losses and to treat newly affected ducks. Sulfamethazine or sodium sulfamethazine was recommended in 0.5 - 1% in the feed or 0.1% in the drinking water. 0.5% sulfamerazine in mash feed for 5 days significantly

reduced mortality, but after withdrawing the treatment, fowl cholera recurred. (25)

Sulfaquinoxaline in the rate of 0.05 - 0.02 %, i,e. 1 : 2000 - 1 : 4000 in the drinking water or 0.1 - 0.05% in the mash for 5 days was usually successful in treating infected flocks and for prophylaxis. The treatment should be started during the early stages of outbreaks. In later stages, when the disease became chronic results were unsatisfactory. Often recurrence of mortality occured after treatment was discontinued. In such cases an intermittent treatment (2 - 3 days treatment, and 2 - 3 days pause) was advisable. (25)

Antibiotics, used in small doses for growth promotion, were not effective in prophylaxis or treatment of fowl cholera. Chlortetracycline and oxytetracycline 500 g/ton of mash feed was effective in experimental conditions and in field outbreaks as well. But in half the cases losses recurred after withdrawal of the antibiotic. (25)

In farms, where fowl cholera occured repeatedly a prophylactic vaccination was recommended. Breeder or layer flocks should be vaccinated twice, first in 2 - 3 month age then 1 - 2 month later. A 6 months immunity might be expected from two vaccinations. If necessary, booster vaccination every 6 months would prolong the immunity. (25)

Fowl cholera, caused by virulent P. multocida organisms, was an infectious disease. The most effective way of protecting flocks against fowl cholera was to prevent the introduction of the infectious agent. As fowl cholera was usually not egg transmitted the introduction of the virulent Pasteurellas was dependent mainly on the owner, who should follow the rules of hygenic management. (25)

Sick birds, or recovered ducks should not be introduced in a flock of ducks, as these might be carriers of virulent organism. Only young birds, under 4 weeks of age, should be introduced as new stock. The different age group should be kept in separate houses. Pigs, dogs, cats and wild birds might be carriers. They should be kept away. The water in canals, lakes, and ponds might contain Pasteurella; and therefore ducks should not be allowed to infected waters. Water supply should be provided from a well or from the main supply. (25)

If an outbreak of fowl cholera occured in a flock, it should be quarantined and slaughtered as soon as economically feasible. A new flock could be introduced only after a thorough deinfection. (25)

1.7 Production of vaccine (32)

1.7.1 Selection of strains

At present this is based on trial and error. Certain strains which were known to immunize effectively were used in many laboratories. It was possible that better strains existed in nature and in the near future these might become detectable by simple antigenic analysis. When selected on the basis of their yield of immunizing antigen they could be grown in media designed for maximum development of these antigens. (32)

1.7.2 Preservation of seed

Pasteurellas could change in two ways during subcultivation. They altered phenotypically in response to the environment, giving varying yields of their different antigens, or they might alter genotypically and lose some antigens altogether. It was a reasonable assumption that the ideal pasteurella was the one which grew in the animal body in the course of infection. This was the kind of bacterium that the animal had to combat

and hence its antigenic pattern should be maintained as nearly as possible in any vaccine. (32)

1.7.3 Killing cultures

Formalin is used universally. It is cheap and effective and is a good preservative as well as a bactericidal agent. Merthiolate and antibiotics produced satisfactory killed suspensions for short term use but they had no advantage over formalin. Killing by heat was detrimental to the vaccine. Formalin is probably effective because of its fixative and toxoiding action on the important protein antigens. (32) Formalin - killed cultures commonly became inagglutinable in antisera and for agglutination tests merthiolate was better. (32)

1.7.4 Types of vaccine available

For practical purposes a vaccine was considered worthwhile only if it could be easily made and distributed and produced lasting immunity from a single injection of small volume without undesirable reactions in the recipient animals. Problems of distribution in Asia weigh against live attenuated vaccines at present, even if a stable reliable one was available. Freedom from adverse reactions such as shock, pain or swelling were most desirable in a vaccine, as these faults were not viewed kindly by populations which were just being convinced of the value of prophylactic vaccination. Effectiveness, stability, safety and blandness were the desired properties of a vaccine to which might be added simplicity and economy in its production. (32)

1.7.4.1 Plain Bacterin

In one form or another bacterins had been in use against pasteurella infection for eighty years. In fact, a dead fowl

cholera culture on Pasteur's workbench was the forerunner of all bacterial vaccines.Despite this illustrious beginning, however, they had never performed very well in the field against any of the animal pasteurellosis unless given in large doses at fairly close intervals of time. The only logical use for bacterin nowadays is for vaccination in the face of outbreaks. (32)

1.7.4.2 Delpys' Vaccine

This consisted of agar - grown pasteurellas lysed in distilled water or with the aid of pepsin and finally combined with saponin as an adjuvant. A strain must be selected which lyses readily and which was not toxic. Contamination was prevented by merthiolate. It was made on a large scale in Iran but it had not been adopted in other countries as its production required the tedious process of cultivation on agar. (32)

1.7.4.3 Alum - precipitated Vaccine

This was prepared very simply from broth bacterin. It was not free from shock reaction so that some cautions were needed with large doses. The method was to add enough of a 10% potash alum solution to formalized suspension to give a concentration of 1% alum in the vaccine. It might be necessary to adjust the pH to produce flocculation. Usually this meant adding alkali to bring the pH to about 6.5. The vaccine was injected subcutaneously in the same dosage as plain bacterin. Some reactions occured at the site of inoculation. (32)

1.7.4.4 Oil - adjuvant Vaccine

This was designed to retard absorption of the vaccine and prolonged the antigenic stimulus to the antibody - forming cells. The

bacterial suspension, killed by formalin, was emulsified in light mineral oil using a stabilizing agent suitable to a water - in - oil emulsion. Vegetable oils were less effective than mineral oils. The emulsion must be of the water - in - oil type so that the droplets of bacterial suspension were distributed throughout the oil. A reverse emulsion would be useless as the bacteria would be absorbed quickly leaving only inert oil at the site. Emulsions must be made with care so that the individual droplets of suspension in the disperse phase were small enough and sufficiently stabilized to prevent coalescence. Coarse emulsions were absorbed too rapidly with loss of prolonged stimulating effects. (32)

Since Pasteur (1880) made the first bacterial vaccine to combat fowl cholera, its success have been the basis of other studies that laid the foundation of our knownlege of artificial immunization against infectious disease. The Pasteur vaccine consisted of living cultures of two grades of virulence. It was administered as a prophylactic measure in two doses, a few days apart, the first being less virulent than the second. This type of vaccine was no longer used.

Pasteur (1880) developed a live vaccine, an attenuated P. multocida vaccine, that worked under laboratory conditions, but behaved erratically under field conditions. (33) Sinha et al (1957) found that a living, nonlethal, highly capsulated mouse avirulent strain of Pasteurella was a better immunizing agent than heat - killed strain. (34)

Recently, development of a live vaccine utilizing an avirulent strain and administered in drinking water has shown promise in field use under a variety of conditions and in widely separated regions.

Hyperimmune serum could provide passive immunity for the immediate protection of animals but antibiotic medication was preferred. (35)

2. Preparation of capsular polysaccharide

2.1 Definition and demonstration of capsular polysaccharide

The capsule was a gelatinous, extracellular layer (thickness $\leq$ 200 mμ) composed of either protein or polysaccharide, which could be resolved by the light microscope. It was not essential for cell viability and was chemically and immunologically different from the cell wall. (36,37)

Capsular polysaccharides could be demonstrated by a variety of staining techniques. The periodate - Schiff stain and the Alcian blue stain had been suggested for the specific demonstration of capsular polysaccharides, the latter dye staining the capsule without preliminary mordanting. Capsules were best demonstrated by techniques in which the capsule was outlined while in a wet (unshrunken) state. Thus, in the wet - film Indian ink method, which Duguid (1951) considered the most reliable, informative, and generally applicable method, the size and shape of the capsular material were outlined by particles of Indian ink unable to penetrate the gelationous capsule. (36,37)

Structural details in unstained living cells might be observed by phase contrast microscopy, which saved time and damage to the cell. (36,37)

Most capsular and slime substances had a very low affinity for various dyes and electron microscopy had established that in general they were less electron - dense than the cell wall and cytoplasm. (36,37)

Negative staining was probably the simplest and quickest technique used. It was useful in the studying of tiny objects such as virus, bacteria. This method consisted of placing the specimen in a drop of

"stain" such as phosphotungstic acid. The metal atoms penetrated between and around the smallest spaces and structurer of the specimens and revealed images not seen by other methods. The space would appear well defined in negative contrast. (38) When viewing in the electron microscope, the particles appeared as light areas due to their low scattering power, surrounded by the electron dense stain. This was the opposite of positive staining which made particles visible by actually combining a heavy metal salt with them. (39)

2.2 General properties of capsules

In mice, the capsule seemed to be a potent contributing factor to virulence and the effective immunity against these organisms was associated with antibodies directed against this capsular material. Other factors, as yet unidentified, might also affect the virulence of this bacterium for mice. (40)

There were some evidences to show that the capsular antigen of P. multocida may confer protection. (40)

The organism usually possessed a capsule when recently isolated from disease processes. Capsules vary greatly in size and composition. The presence of hyaluronic acid in the large capsules of some strains was demonstrated independently by Carter and Bain (1960). (4) Virulent strains might lose their capsular material after continued subculture on artificial media.

Properties of the capsular antigen suggested that it was a high molecular weight acidic polysaccharide. (41)

Capsular antigen was free from endotoxic activity. Capsular antigen was stable when boiling for 10 minutes (min.) or autoclaving at 121 °C for 10 min. (41)

No effects of digestion were discernible when the antigen was digested with pronase. Capsular antigen was dissolved in 0.05 M. tris - acetic buffer, pH 8.0 to a concentration of 10 milligrams per milliliter (mg/ml). Pronase (2 mg/ml) was added and the mixture was incubated at 37 °C for 18 hours (hr). (41)

Separation of the capsular antigen and endotoxin from saline extracts of P. multocida type B was achieved by fractional precipitation from aqueous solution by addition of polar organic solvents. Biological tests for the presence of endotoxin shew that it was absent from capsular antigen preparation so obtained. The solvent fractionation method was found to be equally applicable to separation of capsular antigen and endotoxin of P. multocida type E. (41)