WAR MEMORIAL DOORWAY

This book is the property of
MacNabb Memorial Library
Ontario Veterinary College
A SEROLOGICAL CLASSIFICATION OF
PASTEURIELLA MULTOCIDA

by

Gordon Robert Carter

A thesis presented to the Faculty of the Graduate School
of the University of Toronto in fulfilment of
the requirements for the degree of

Doctor of Veterinary Science

January 1956
# CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td><strong>Part I</strong></td>
<td></td>
</tr>
<tr>
<td>The Type Specific Capsular Antigen of Pasteurella Multocida</td>
<td>8</td>
</tr>
<tr>
<td>Literature Review</td>
<td>9</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>15</td>
</tr>
<tr>
<td>Results</td>
<td>21</td>
</tr>
<tr>
<td>Discussion</td>
<td>22</td>
</tr>
<tr>
<td><strong>Part II</strong></td>
<td></td>
</tr>
<tr>
<td>Isolation of Capsular Polysaccharides from Colonial Variants of Pasteurella Multocida</td>
<td>24</td>
</tr>
<tr>
<td>Literature Review</td>
<td>25</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>27</td>
</tr>
<tr>
<td>Results</td>
<td>30</td>
</tr>
<tr>
<td>Discussion</td>
<td>33</td>
</tr>
<tr>
<td><strong>Part III</strong></td>
<td></td>
</tr>
<tr>
<td>A Haemagglutination Test for the Identification of Serological Types of Pasteurella Multocida</td>
<td>37</td>
</tr>
<tr>
<td>Literature Review</td>
<td>38</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>40</td>
</tr>
<tr>
<td>Results</td>
<td>44</td>
</tr>
<tr>
<td>Discussion</td>
<td>48</td>
</tr>
<tr>
<td><strong>SUMMARY OF PARTS I, II AND III</strong></td>
<td>50</td>
</tr>
</tbody>
</table>
INTRODUCTION

The importance of *Pasteurella multocida* as an animal pathogen has been appreciated for many years. Its host range is remarkably broad. In recent years in central Canada *P. multocida* has been isolated from cattle, swine, sheep, goats, dogs, cats, rabbits, chickens, turkeys, ducks, chinchilla, mink, mice and guinea pigs. Reports of isolations from human beings appear occasionally in the literature. The distribution of this important parasite is worldwide.

*P. multocida* is harboured in the respiratory passages of a considerable proportion of apparently normal domesticated animals. Some indication of the carrier rate is given in Table 1. It is not surprising in view of the high incidence of carriers that *P. multocida* occurs so frequently in disease involving the respiratory system.

In years past the isolation of *P. multocida* from a diseased organ or tissue was considered sufficient evidence to attribute to it a primary aetiological role. However, in view of the rapid progress of animal virology in recent years it has become necessary to reassess the role of *P. multocida* in animal disease. Much of the available information on pasteurella infections was based upon reports which were published several decades prior to the extensive study of virus infections of animals. In this introduction it is planned to review briefly the present status of *P. multocida* in animal disease in the
Cattle and Buffalo

The most important pasteurella infection in these species is the well known haemorrhagic septicaemia. The term haemorrhagic septicaemia denotes an acute pasteurella infection usually appearing as an epizootic. The disease which appeared several decades ago in the buffalo of Yellow Stone National Park was no doubt epizootic pasteurellosis or haemorrhagic septicaemia (21). This disease occurs frequently in South Asia and is responsible for enormous losses of cattle and buffalo (4). In India it is considered the most important bacterial disease of cattle and buffalo (49). Serious epizootics of haemorrhagic septicaemia in cattle and buffalo have been reported frequently from tropical and subtropical countries.

Some years ago a controversy arose in the British veterinary press over whether haemorrhagic septicaemia existed in Great Britain (20, 56). The status of haemorrhagic septicaemia on this continent was raised recently by Aitken (1). In the author’s experience haemorrhagic septicaemia does not exist as an epizootic disease in Canada. It seems very likely that such is also the case in the United States, the British Isles and Northern Europe. Acute fatal infections characterized by a haemorrhagic septicaemia are seen occasionally in central Canada as sporadic infections.

*P. multocida* is frequently recovered from the pneumonic lungs of cattle. Pneumonia may occur in one or several animals, or an outbreak such as transit or shipping fever may be observed (12).
Recent work has shown that *P. multocida* and *P. haemolytica* are the principal bacterial agents in shipping fever in Canada (12, 13). Serological evidence that infections with *P. multocida* take place during or shortly after shipment of cattle from Western Canada has been obtained by Rice and associates (50). Frequently *P. multocida* complicates already established chronic pneumonias.

*P. multocida* is not infrequently recovered from the udders of cows with mastitis. Barnum (5) has described a herd outbreak of *Pasteurella* mastitis. Isolations of *P. multocida* have been made from diverse disease processes in cattle including abscesses and encephalitis.

**Fowl**

Fowl cholera is an important disease with worldwide distribution. It is thought that it occurs less frequently now in the north temperate zone than it did several decades ago. The improvement in general poultry management practices is cited as the reason. Cholera continues to be a major disease problem of fowl in many tropical and subtropical countries. That *P. multocida* is the primary cause of fowl cholera is apparently not disputed.

*P. multocida* is frequently recovered from various chronic processes in fowl. Fahey (19) has reported the isolation of *P. multocida* from "air sac infection" of chickens a disease the primary cause of which is believed to be a pleuropneumonia-like organism.

**Swine**

Although haemorrhagic septicaemia has been described as
an important disease of swine in years past it no longer appears to be a prevalent disease on the North American continent. One even doubts if such a disease exists as an epizootic of swine in Canada and the United States. Reports of epizootic haemorrhagic septicaemia are almost nonexistent in the current literature. It seems likely that the disease which we now refer to as virus pneumonia of pigs (VPP), was once thought to be a pasteurellosis of swine. *P. multocida* is certainly the most important secondary invader in VPP (14) in Canada. Because it is present in a secondary capacity there has been a tendency to belittle its importance in VPP. From the standpoint of pathological activity it is probably more important than the virus; however, in regard to elimination of the disease from a herd the virus is all important.

*P. multocida* is no doubt an important organism in infectious atrophic rhinitis. Its precise role in this enigmatic disease has not yet been determined (24).

Sheep

Haemorrhagic septicaemia is described in many current texts as occurring in sheep. This disease does not appear to occur in Canada and reports of its occurrence have not appeared in the American or European literature in recent years.

As in cattle pulmonary infections are not infrequent. They are usually sporadic but may appear as herd outbreaks. These respiratory infections are essentially a bacterial bronchopneumonia as opposed to the acute haemorrhagic septicaemia. Whether or not
P. multocida is primary in these infections is not known.

Man

P. multocida has been isolated from a variety of pathological processes in human beings. Olsen and Needham (41) suggest that pasteurella infections probably occur more frequently in human beings than is realized. The most frequent infections in man are those involving the respiratory tract and those resulting from cat and dog bites. The clinical diagnosis in 37 cases of human pasteurellosis (P. multocida) reported by Olsen and Needham is given in Table 2.

Other Species

Infections in horses, goats, dogs, cats and other species are generally believed to be sporadic. Sufficient information is not available at present to state whether or not these infections are primary or secondary.

The foregoing brief review of the pathogenic status of P. multocida made no reference to the occurrence of different serological types or varieties in the various disease manifestations for the very good reason that adequate information on this matter is not available. Such information would not only increase greatly the knowledge of the epidemiology of pasteurellosis but would allow for the production of prophylactic antisera and bacterins along rational lines.

It is the purpose of the studies reported in this thesis to obtain first, sufficient information on the antigenic nature of
strains of *P. multocida* to make possible a serological classification of the species; and second to determine the distribution of the different serological types in the various disease manifestations in the different principal domesticated animals.
Table 1

Incidence of *Pasteurella multocida* in the Respiratory Tract of Healthy Animals

<table>
<thead>
<tr>
<th>Animal</th>
<th>Site Examined</th>
<th>Per Cent Carrier Rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cattle</td>
<td>Nose (28)</td>
<td>15</td>
</tr>
<tr>
<td>Buffalo</td>
<td>Nose</td>
<td>3.5</td>
</tr>
<tr>
<td>Pig</td>
<td>Tonsil, mouth, lymph nodes</td>
<td>51</td>
</tr>
<tr>
<td>Pig</td>
<td>Lungs</td>
<td>26</td>
</tr>
<tr>
<td>Cat</td>
<td>Air passages</td>
<td>75</td>
</tr>
<tr>
<td>Rat</td>
<td>Throat</td>
<td>14 - 17</td>
</tr>
<tr>
<td>Dog</td>
<td>Nose</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>Tonsils</td>
<td>54</td>
</tr>
</tbody>
</table>

* Smith (55), modified.

Table 2

Clinical Diagnosis in 37 Cases of Human Pasteurellosis (Al)

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>No. Cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bronchiectasis</td>
<td>27</td>
</tr>
<tr>
<td>Bronchiectasis and empyema</td>
<td>2</td>
</tr>
<tr>
<td>Bronchiectasis and tuberculosis</td>
<td>1</td>
</tr>
<tr>
<td>Bronchiogenic carcinoma and empyema</td>
<td>1</td>
</tr>
<tr>
<td>Chronic bronchitis</td>
<td>3</td>
</tr>
<tr>
<td>Peritonsillar abscess</td>
<td>1</td>
</tr>
<tr>
<td>Appendiceal abscess</td>
<td>1</td>
</tr>
<tr>
<td>Infection of gouty joint</td>
<td>1</td>
</tr>
</tbody>
</table>
PART I

THE TYPE SPECIFIC CAPSULAR ANTIGEN

OF PASTEURELLA MULTOCIDA
Part I

The Type Specific Capsular Antigen of Pasteurella multocida

The primary purpose of this part of the investigation was to obtain sufficient knowledge of the antigenic structure of *P. multocida* to make possible a satisfactory technique for the recognition of different serological types. The serological methods which have been applied to *P. multocida* in the past have usually depended upon immunological reactions involving both specific and nonspecific factors and consequently strong cross reactions have made difficult the unequivocal identification of types.

**Literature Review**

The literature on *P. multocida* is abundant. In this review only those reports which have contributed more or less directly towards an ultimate serological classification will be referred to.

Lignières (34) described the organism as it is known today and recommended that the genus name *Pasteurella* suggested by Trevisan be employed. He thought that from each of the various pasteurelloses a different species could be recovered. He was thus the originator of the zoological classification of *P. multocida* which has been employed until quite recently. By this classification strains of *P. multocida* were named according to the animal species from which they were isolated, e.g. if recovered from a cow or a calf it was called *P. bovis septica*, from a pig it was named *P. suis septica*, etc.
DeKruif (16) was the first investigator to study in detail the variants of \textit{P. multocida}. He described two variants, viz., diffuse and granular after the manner of their growth in broth. Webster and Burn (42) added to these variants an intermediate and a mucoid type. The terms smooth and rough were introduced by Anderson, Coombes and Mallick (3) to replace the terms diffuse and granular. Hughes (26) referred to the variants he observed as fluorescent, intermediate and blue. The term fluorescent denoted the iridescence observed when colonies on transparent agar were illuminated by oblique light.

Elberg and Cheng-Lee Ho (18) studied the fluorescence and other properties of the different variants of \textit{P. multocida}.

To clarify the confusion which has resulted from the designation of the same variants by different terms all of the variants are listed in Table 3 with the various terms which have been employed to designate them. The principal properties which have characterized the different variants are also summarized in Table 3.

The relationship of colonial variation of \textit{P. multocida} to the epidemiology and pathogenesis of pasteurellosis was studied by a number of workers. Pritchett, Beaudette, and Hughes (47, 48) and Webster and Burn (57) observed that the blue and mucoid variants were frequently recovered from chronic processes and carrier states in chickens and rabbits. The fluorescent and intermediate variants were more frequently isolated from fowl cholera during the epidemic phase. The observations cited above were confirmed by Carter and Byrne (10) and Carter and Bigland (11).
The question of the origin of the mucoid forms was posed by Webster and Bum (58). Elberg and Cheng-Lee Ho were probably the first workers to show that the mucoid variants were derived from the well known fluorescent forms and have worked out the dissociation pattern of several strains in considerable detail. Carter and Bigland suggested that dissociation in P. multocida proceeded as follows:

$$S \rightarrow M \rightarrow R$$

An important contribution to the classification of Pasteurella strains was made by Jones (27) in 1921. He divided 16 bovine strains into three groups on the basis of biochemical and serological relationships. His group I was composed of strains of what are now considered another species, viz. Pasteurella hemolytica. The serological method used was the tube agglutination test. Jones was therefore, probably the first investigator to demonstrate different serological types of P. multocida.

Employing the agglutination test and biochemical criteria Jorgenson (28) divided 37 bovine strains into four groups, three of which were the same as Jones' groups. Newsom and Cross (39) working with strains from both sheep and cattle divided them into typical and atypical groups. The typical group resembled Jones' group I or what is now called P. hemolytica. The atypical group corresponded to Jones' group III. By agglutination tests each of the two groups was divided into two subgroups. It was noted that the same serological type of Pasteurella was recovered from both sheep and cattle.
<table>
<thead>
<tr>
<th>Designation of Variants (synonymous terms listed together)</th>
<th>Colonies on Agar</th>
<th>Growth in Broth</th>
<th>Agglutinability</th>
<th>Mouse Virulence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluorescent (<em>F</em>)* Smooth (<em>S</em>) Diffuse</td>
<td>Medium in size, discrete</td>
<td>Diffuse</td>
<td>Moderate</td>
<td>High</td>
</tr>
<tr>
<td>Mucoid (<em>M)</em></td>
<td>Large, moist, flowing</td>
<td>Diffuse, with mucoid deposit</td>
<td>None</td>
<td>Moderate</td>
</tr>
<tr>
<td>Intermediate (<em>I)</em></td>
<td>Medium in size, discrete</td>
<td>Diffuse</td>
<td>Moderate to marked</td>
<td>High</td>
</tr>
<tr>
<td>Blue (<em>B)</em> Granular Rough (<em>R)</em></td>
<td>Small, discrete</td>
<td>Flocculent with deposit</td>
<td>Marked</td>
<td>Low</td>
</tr>
</tbody>
</table>

* These terms are used in the studies reported below.
The studies referred to above served to disprove the zoological classification advanced by Lignières. Further evidence showing that strains were not necessarily zoologically specific was obtained from a number of successful cross-pathogenicity trials (22, 37).

Employing the complement-fixation test Roderick (52) demonstrated two distinct serological types in 24 strains examined. Cornelius (15) was able to divide 17 of his 26 strains into four groups by agglutination-absorption tests. By precipitin-absorption Musaf (59) confirmed the findings of Cornelius. Ochi (40) and Zaisen (60) employing respectively the agglutination and complement-fixation tests divided their strains into several groups. Khalifa (32) classified strains on the basis of the agglutination test and fermentative activity in xylose and arabinose.

Hoffenreich (25) extracted a polysaccharide substance from the capsules of P. multocida, which resembled in its physical, chemical, and serological properties, the soluble specific capsular polysaccharides isolated by earlier workers from the capsules of a number of pathogens. Employing the well known method of Boivin and Mesrobeanu, Pirosky (42) extracted polysaccharide antigens from smooth forms of P. multocida and by means of precipitation tests he demonstrated their serological specificity and suggested that their specificity might make possible a serological classification of the group.

Rosenbusch and Merchant (53) divided 39 strains into three principal groups on the basis of agglutination, and action on xylose, arabinose and dulcitol. They observed that some strains of P. multocida
were not consistent in their fermentative activity. Strong cross agglutination was observed between their serological groups. Seven of their strains were classified as variants. Little and Lyon (35) found that their 30 strains fell into three distinct groups when typed by means of slide agglutination and serum protection tests. Roberts (51) employed serum protection tests in mice to divide his 37 strains into four principal groups.

In spite of the plethora of work which has been devoted to the classification of this species a satisfactory typing method and a comprehensive scheme of classification had not evolved.
Materials and Methods

Origin and Maintenance of Cultures

The animal origin and proposed serological designation of the four cultures employed to demonstrate the type specific capsular antigen are given in Table 4. All cultures were maintained on air-tight beef-infusion agar slants at room temperature. When it was necessary to maintain cultures for long periods transfers were made at monthly intervals. Only strains conforming with the description of *P. multocida* as given in Bergey's manual (7), except for the production of hydrogen sulphide, were included in this study.

Preparation of Antisera

Only cultures yielding a large proportion of fluorescent and intermediate colonial forms produced sufficient amounts of specific capsular antigen for the preparation of the specific sera referred to in Table 5. Avirulent cultures yielding a large proportion of blue variants on clear tryptose horse serum agar were passed in mice or chick embryos. After one or more passages they were observed to produce a large proportion of fluorescent and intermediate forms. The organisms for immunization were grown on tryptose horse serum (10%) agar containing 0.2% dextrose and 1.0% sucrose. After incubation at 37°C for 24 hours the growth from each plate was washed twice in normal saline and then suspended in sufficient 0.25% formalized buffered saline (pH 7.0) to produce a turbidity corresponding to tube No. 4 of McFarland's nephelometer. Before immunization
Table 4

Sero logical Designations and Animal Source of *P. multocida* strains

<table>
<thead>
<tr>
<th>Culture No.</th>
<th>Serological Designation</th>
<th>Animal Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>1000</td>
<td>A</td>
<td>Buffalo</td>
</tr>
<tr>
<td>1001</td>
<td>A</td>
<td>Bovine</td>
</tr>
<tr>
<td>1002</td>
<td>B</td>
<td>Buffalo</td>
</tr>
<tr>
<td>1003</td>
<td>C</td>
<td>Canine</td>
</tr>
</tbody>
</table>
Table 5

Results of Precipitation Tests Demonstrating the Specificity of the Capsular Antigen of *P. multocida* Strains

<table>
<thead>
<tr>
<th>Serum</th>
<th>Antigen</th>
<th>1:2</th>
<th>1:4</th>
<th>1:8</th>
<th>1:16</th>
<th>1:32</th>
<th>1:64</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>1000</td>
<td>1000</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Type</td>
<td>1001</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>A</td>
<td>1002</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>1003</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1002</td>
<td>1000</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Type</td>
<td>1001</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>B</td>
<td>1002</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1003</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1003</td>
<td>1000</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Type</td>
<td>1001</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>C</td>
<td>1002</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>1003</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td></td>
</tr>
</tbody>
</table>

Note: ++ = Precipitate.
+ = Slight precipitate.
was begun the rabbits and chickens employed for the preparation of immune sera were tested for the presence of precipitins against *P. multocida*.

Satisfactory titers were obtained in some rabbits after 12 intravenous injections. Rabbit antisera were employed for the precipitation tests referred to in Table 5. Sera from several chickens were used for the capsular swelling tests after the administration of six to eight inoculations of antigen. With rabbits the immunizing dose ranged from 1.0 ml. to 5.0 ml. and for chickens a uniform dose of 3.0 ml. was administered. Several additional injections of living organisms administered in decreasing dilutions were required to produce antisera sufficiently potent for MacPherson's (26) precipitation test. The intervals between injections varied from three to seven days.

**Precipitation Tests**

(1) This method was adapted from Pittman's (45) technique of demonstrating the type specific capsular antigen of *Haemophilus influenzae*. The antigen for this test consists of what is presumed to be soluble polysaccharide material, released by capsulated organisms when suspended in normal saline. Because the capsular material of mucoid strains appeared to be less soluble in saline than that of fluorescent strains, only nonmucoid strains were typed by this test. Each culture listed in Table 4 was inoculated heavily on a tryptose horse serum agar plate. After incubation at 37°C. for 24 hours, the growth was washed off with 3.0 ml. of buffered saline.
and the suspension heated at 56°C for one hour. The organisms were centrifuged out and by the method of doubling dilutions the supernatants were diluted to a final dilution of 1:64 in buffered saline. The antisera in 0.2 ml. amounts were added to 0.2 ml. amounts of the supernatant dilutions and the mixtures incubated at 37°C for one hour and then left at 4°C for 18 hours prior to reading. A positive test consisted of a precipitate which could be readily discerned when the tubes were shaken sharply. Control tubes contained 0.2 ml. of the buffered saline diluent and 0.2 ml. of the specific antisera. Data in regard to the dilutions and identity of antisera and antigens are given in Table 5.

(2) The technique evolved by MacPherson for typing *H. influenzae* separated the strains listed in Table 4 into the same categories as the aforementioned method and had the advantage of involving the use of considerably less serum. All strains producing fluorescent, intermediate, and blue colonies on agar were typable by this method. By this method MacPherson was able to type nontypable (by the capsular swelling technique), nonencapsulated, derivative strains of *H. influenzae*. The technique employed was identical to that described by MacPherson except that the cultures of *P. multocida* were cultivated on tryptose horse serum agar rather than on Fildes or Levinthal agar. As in the previously described test the antigen consists of the type specific soluble capsular substance.

**Capsular Swelling Tests**

Capsulated organisms for this test were obtained from 0-15
hr. tryptose broth cultures and from 24 hr. 10-day chick embryo cultures. Hanging drop mounts were prepared as follows: 1. A ridge of petrolatum in the outline of the perimeter of a cover slip was applied to a glass microscope slide with a hypodermic syringe. 2. Two loopfuls of a given antiserum were mixed with a loopful of Loeffler's methylene blue solution on a glass slide. 3. A bacterial suspension was prepared by the addition of one or two drops of the broth, or the allantoic fluid of a chick embryo culture to 5.0 ml. of normal saline. A small loopful of this suspension was added to the antiserum-methylene blue mixture. After thorough mixing, one loopful was transferred to a cover slip which was inverted over the perimeter of petrolatum thus giving a hanging drop. The examinations of capsular swelling were greatly facilitated by the use of double-size cover slips from which were suspended several drops each containing a specific antiserum. 4. Microscopic examinations of the moist preparations were made after the mixtures had stood at room temperature for periods of 15 and 30 min. Antisera were diluted to overcome cross reactions between different types. As swelling takes place the capsule becomes more prominent and opaque, and its outer edge becomes more clearly outlined.
Results

The results of the precipitation tests in which the capsular antigen was washed from the organisms are shown in Table 5 and they demonstrate very clearly the type specificity of the capsular antigen. The type identity of the strains listed in Table 4 was also demonstrated by capsular swelling tests and by MacPherson's precipitation test.
Information derived from this investigation and from the studies of other workers throws considerable light on the antigenic nature of P. multocida. There appear to be two principal antigenic components: 1. A type specific, soluble polysaccharide antigen consisting of or associated with the capsule, and 2. a somatic antigen common to all members of the species.

That the capsular antigen is polysaccharide in nature may be assumed from the following evidence: The identification by Höffnerich of a polysaccharide substance associated with the capsule. The tendency of capsules to swell in the presence of homologous antiserum. MacPherson's technique for typing H. influenzae by the extraction of specific polysaccharide has been employed with P. multocida and has yielded results identical with those obtained with the first technique described in this study.

Considering that it is the capsular antigen which elicits the greatest immune response in mice (46), one would expect a close correlation between type and immunological specificity. That such is the case has been demonstrated by vaccination and challenge experiments in mice (9).

The difficulties which have been encountered in the past in the typing of P. multocida appear to have been due for the most part to the complex dissociation pattern of the organism. P. multocida
may yield in the same culture varying numbers of fluorescent, intermediate, and blue variants. Cultures containing a large proportion of fluorescent forms are almost inagglutinable although, because they possess large amounts of capsular antigen, they are highly satisfactory for Quellung tests. Strains producing flocculent growth in broth, a large number of blue variants on agar, and manifesting low virulence for mice and chick embryos, often undergo spontaneous agglutination and yield a very small amount of specific capsular antigen. Antigens prepared from these cultures were found to be unsatisfactory for both agglutination and precipitin tests. Because mucoid strains, as compared with fluorescent strains, are generally less virulent, they are difficult to type by means of serum protection tests in mice.

Because Roberts, Rosenbusch and Merchant, and Little and Lyon have employed different numbers to designate what appear to be identical types, and because it is felt that the methods of typing described in this study are less subject to error than previous techniques described, a new alphabetical designation is employed.
PART II

ISOLATION OF CAPSULAR POLYSACCHARIDES FROM COLONIAL VARIANTS OF PASTEURELLA MULTOCIDA
Part II

Isolation of Capsular Polysaccharides from Colonic Variants of Pasteurella multocida

In Part I of these studies a type specific substance was extracted from *P. multocida* by heating the organisms in normal saline at 56°C for an hour. The purpose of this portion of the studies is to describe the method of isolation, and some of the chemical and biological properties of the soluble capsular substances. If there were a difference in the chemical nature of the capsular substances of the fluorescent and mucoid variants, it would account for some of the difficulty that has been experienced in typing *P. multocida*.

**Literature Review**

Hoffenreich (25) reported the isolation of a capsular polysaccharide from *Bacillus avisepticus* possessing properties similar to the well-known soluble specific substances of the pneumococcus. This polysaccharide was serologically active, had a nitrogen content of 1.2%, and after hydrolysis yielded 46% reducing substances.

Dingle (17) recovered a polysaccharide from *Bacterium lenisenticum*. It was a white powder, readily soluble in water and active serologically in dilutions as high as 1:200,000. Some years later Pirosky (42) recovered two distinct glycolipid antigens from 'smooth' and 'rough' forms of *P. aviseptica*. Kendall, Heidelberger and Dawson
(30), and Seastone (54) isolated serologically inactive capsular polysaccharides from mucoid group C streptococci.
Materials and Methods

Cultures

These were: strain 41 type A (buffalo), 312 type B (bovine) and 398 type C (canine). Culture 312 was freshly isolated from a case of calf pneumonia. The other strains of *P. multocida* were isolated a number of years before this study was begun.

Serological Procedures

Rabbit sera were prepared from all cultures in the manner outlined in Part I. A precipitin test was employed in which 0.1 ml. amounts of antigen dilutions were layered over 0.1 ml. amounts of undiluted antisera. The tubes were incubated for one hour at 37°C, then read. The presence of a white precipitate in the form of a thin ring at the junction of the antigen and antiserum constituted a positive reaction.

Cultivation of *Pasteurella multocida*

Organisms were cultivated on beef infusion agar contained in oversize Petri plates (diameter 15 cm.). Thirty to 35 plates were employed for each preparation. Polysaccharides were recovered from cultures 41 and 312, but those recovered from the latter were studied in the greatest detail. It was possible to some extent to produce cultures possessing predominantly I variants, F variants, or M dissociants by altering the constituents of the culture medium.

In order to obtain the I variant, culture 312 was injected into a mouse, intraperitoneally. At 15 to 18 hours after injection, heart's blood was removed aseptically and a few drops were inoculated.
into each of several tubes of beef infusion broth. After eight to 15 hours' incubation, each Petri plate containing beef infusion agar was seeded with 0.2 ml. of broth. The F variant was obtained in the same manner except that the beef infusion agar contained 0.3% sucrose and 0.3% dextrose. To produce the M variants, culture 312 was not passed in a mouse, the broth cultures being seeded direct from an agar slant. After incubation the Petri plates containing beef infusion agar supplemented with 0.3% sucrose and 0.3% dextrose were seeded with 0.5 ml. of broth. The growth was washed off with cold distilled water after incubation for 15 to 18 hours. Great care was taken to eliminate agar from the harvest of organisms. Culture 312 differed from cultures 41 and 393 in that it produced mucoid forms when handled as just described.

**Isolation of Polysaccharides**

The harvested suspensions were heated in a water bath at 56°C. for one hour, after which the bacteria were sedimented by high speed (10,000 r.p.m.) centrifugation. The slightly opalescent, amber supernatant fluid was reduced to 15-20 ml. by vacuum distillation and treated with 3 volumes of 95% ethyl alcohol containing 0.25% sodium acetate. A viscous, stringy precipitate appeared which was collected by the rotation of a glass rod and/or centrifugation. The viscous precipitate was rubbed in a small amount of distilled water gradually increasing to 10 ml. This was centrifuged at high speed to sediment insoluble material. The whole procedure was repeated and the final alcoholic precipitate, the third, was sedimented by centrifugation.
It was then washed with absolute alcohol and dried in vacuo over cal-
cium chloride. Prior to conducting chemical analyses the polysacchar-
ides were further dried over phosphorus pentoxide in a drying chamber
at 65°C.
Results

Polysaccharides possessing different chemical and biological properties were isolated from each of the three dissociation phases, i.e., the I, F, and M variants. Nine preparations of polysaccharides from culture 312 were studied. Some of their properties are presented in Table 6.

The figures in regard to yield and chemical composition represent averages of the values obtained from several preparations. The differences in the values obtained from the chemical analyses of the various preparations from the same variant were in the range of 1 to 2 per cent. Solutions of unhydrolyzed substances gave negative Fehling and biuret tests, and positive Molisch (for hexoses) and Bial (for pentoses) reactions. Reducing substances were liberated after hydrolysis with 2 N HCl in sealed ampoules in a boiling water bath for three hours. The presence of hyaluronic acid in the M substance was demonstrated by the action of hyaluronidase. The depolymerization of the polysaccharide was determined by the turbidimetric method of Kass and Seastone (29). The yield of dried bacilli remaining from each preparation after extraction was approximately 500 mg.

Physical Characteristics

The polysaccharides of the three different variants possessed somewhat different physical characteristics, although, in general, the F and I substances were similar. In the desiccated state, the latter were white to cream in color, and flaky and horny in consistency. The dried M substance was white and consisted of white, fluffy powder
possessing numerous fine filaments. It dissolved readily in distilled water and a 0.1% solution possessed a slight opalescent cast. The F and I substances in 0.1% solutions were less soluble and displayed more opalescence.

**Sero logical Specificity**

The results of the precipitation tests conducted with F and M substances are presented in Table 7. Although, in some instances, there were cross reactions, a considerable degree of specificity was displayed.

**Immun ore nicity**

An experiment was conducted to determine whether or not the two polysaccharides were immunogenic in mice. One group of mice (Swiss albino, 18 to 22 Gm.) was inoculated with M polysaccharide while another was injected with the F substance. Three 0.5-ml. doses of 0.01% saline solution was administered intraperitoneally to each mouse. The injected and control group were challenged nine days after the last inoculation with 0.5 ml. of various dilutions of an 18-hour culture of strain 312. The results are presented in Table 8.

The 16 mice surviving were divided into two groups, each consisting of nine mice. One group was rechallenged with 0.5 ml. of a $10^{-5}$ dilution of strain 398 type C, and the other with the same amount of culture 41 type A. Repeated titrations in mice have shown the l.d. 50 of these two cultures for mice to lie between 0.5 ml. of a $10^{-8}$ and 0.5 ml. of a $10^{-9}$ dilution. All of the mice in both groups succumbed to infection. The results of these experiments indicate
that the F polysaccharide is immunogenic and immunologically specific. The M substance elicited no appreciable immune response in the above experiment.

Toxicity

Doses of the M and F polysaccharides (culture 312) in amounts varying from 0.5 to 3.0 mg. were inoculated intraperitoneally into adult mice. There was no reaction other than evidence of considerable abdominal distress for several hours after inoculation.
Discussion

The values obtained for reducing substances and nitrogen differ considerably with those reported by Hoffenreich. However, a perusal of chemical data on various bacterial polysaccharides discloses considerable variation in the results obtained by different workers studying the same bacterial species by different methods (8).

The glycolipid of *P. aviseptica* isolated by Pirosky (44) appears to be distinct from the polysaccharides described in this study. That they are somatic antigens only is strongly suggested by their chemical nature and the fact that they were isolated by the technique employed widely by Boivin for the isolation of the well-known somatic glycolipid antigens. These "Boivin antigens" of *P. aviseptica* differ also from the capsular polysaccharides in that they were highly toxic, the l.d. ⁵₀ for mice being in the range of 0.05 to 0.1 mg.

Pirosky (42) implied that *P. aviseptica* did not possess a capsular antigen distinct from the glycolipid antigen. Such an inference may be attributed to the fact that his cultures were probably in an acapsular or blue phase as is often the case with old laboratory strains. That such was the case is suggested by his reference (43) to the avirulence for mice of his challenged culture.

Strains of *P. multocida* isolated from acute pasteurellosis usually produce many F colonies and a few I variants. It would appear that a preponderance of I variants rather than F forms was produced on beef infusion agar probably because of a limited supply.
Table 6

Properties of Polysaccharides Isolated from Different Dissociants of Bovine Culture 312, a Type B, Pasteurella Multocida

<table>
<thead>
<tr>
<th>Sources of polysaccharides</th>
<th>Predominantly</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>I variants</td>
</tr>
<tr>
<td>Yield</td>
<td>14.0 mg.</td>
</tr>
<tr>
<td>Nitrogen*</td>
<td>5.7 %</td>
</tr>
<tr>
<td>Reducing substances**</td>
<td>13.6 %</td>
</tr>
<tr>
<td>Phosphorus</td>
<td>4.6 %</td>
</tr>
</tbody>
</table>

* Micro-Kjeldahl; ** Somogyi-Shaffer-Hartmann; Fiske-Subba Row.

Table 7

Precipitation Tests Demonstrating the Serological Specificity of the F Substance and the Serological Inactivity of the M Substance of Pasteurella Multocida

<table>
<thead>
<tr>
<th>Serum</th>
<th>Polysaccharide antigen</th>
<th>Saline dilutions of antigens</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>10^{-3}</td>
<td>10^{-4}</td>
</tr>
<tr>
<td>41 (F and I) Type A</td>
<td>41(F)*</td>
<td>++**</td>
<td>+</td>
</tr>
<tr>
<td>312 (F) Type B</td>
<td>312(F)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>312 (M)</td>
<td>312(M)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>398 (F and I) Type C</td>
<td>41(F)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>312 (F)</td>
<td>312(F)</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>312 (M)</td>
<td>312(M)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>312 (M) Type B</td>
<td>312(M)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>312(F)</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

* Variant source; ** very marked ring; distinct ring.
Table 3
Immune Response in Mice to Injections of the M and F Poly-
saccharides of Pasteurella multocida as Indicated by
Homologous Challenge

<table>
<thead>
<tr>
<th>Injected with</th>
<th>$10^{-5}$</th>
<th>$10^{-6}$</th>
<th>$10^{-7}$</th>
<th>$10^{-8}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>M substance (strain 312)</td>
<td>5/5</td>
<td>...</td>
<td>5/5</td>
<td>4/5</td>
</tr>
<tr>
<td>F substance (strain 312)</td>
<td>1/7</td>
<td>0/6</td>
<td>0/6</td>
<td>...</td>
</tr>
<tr>
<td>Control groups</td>
<td>5/5</td>
<td>5/5</td>
<td>5/5</td>
<td>4/5</td>
</tr>
</tbody>
</table>

Numerator = died; denominator = number challenged.
of carbohydrate. That the difference between the F and I variants may be more quantitative than qualitative in nature is suggested by the fact that both substances displayed marked serological specificity. An excess of carbohydrate and surface moisture appeared to exert a selective action for M variants.
PART III

A HAEMAGGLUTINATION TEST FOR THE
IDENTIFICATION OF SEROLOGICAL TYPES
OF PASTEURELLA MULTOCIDA
Part III

A Haemagglutination Test for the Identification of Serological Types of Pasteurella multocida

It became evident after an extensive study of a large number of strains of *P.* multocida that the serological procedures described in Part I had certain shortcomings as routine typing methods. Strong cross reactions were observed frequently with the capsular swelling test and the precipitation methods required large amounts of immune serum. For these reasons a more satisfactory serological procedure was sought.

In view of recent work on the adsorption of bacterial polysaccharides to erythrocytes and the subsequent agglutination of the treated red blood cells by specific antibody, it was thought that this procedure might lend itself to the identification of serological types of *P.* multocida. In this portion of the studies a haemagglutination technique is described which has given results exceeding in specificity any of the previously employed typing methods.

Literature Review

Keogh, North and Warburton (31) first reported the adsorption of the type specific polysaccharide of *Haemophilus influenzae* to erythrocytes and their subsequent agglutination in the presence of homologous immune serum. Since that time numerous reports have appeared describing the adsorption of antigenic polysaccharides to erythrocytes. It is of interest that of the antigenic substances
only polysaccharides appear to attach themselves to erythrocytes.
Before proteins can be adsorbed the erythrocyte receptors must in
some way be modified. Boyden (6) has accomplished this modification
by treating erythrocytes with tannic acid.

Dingle (17) has shown that some strains of *P. multocida*
possess heterophile antigens. For this reason sheep red blood cells
are agglutinated by some *P. multocida* antisera. Alexander, Wright
and Baldwin (2) found that type 0 human erythrocytes were the most
satisfactory for the adsorption of a polysaccharide antigen from
*Pasteurella tularensis*. Landy (33) has discussed the various haem-
agglutination procedures in a recent review.
Preparation of Extracts of Pasteurella multocida

The 18 to 24 hour confluent growth from a blood agar plate (7-8% horse blood or a fresh beef infusion agar plate) was removed with 4.0 ml. of normal saline. It was heated at 56°C. for 30 minutes to assist the removal of the capsular antigen. The organisms were then separated by centrifugation and the supernatant transferred to another tube.

Preparation of Human Type 0 Erythrocytes

Human type 0 red cells were obtained through the courtesy of local hospitals. They were used with satisfactory results for periods up to four weeks after collection. The cells were stored in the original transfusion bottles and sufficient blood for three days was drawn off aseptically with a syringe. These samples were washed three times with six volumes of saline solution. Packed cells from the last washing were stored in the refrigerator and used as desired for a period of three days. Sheep and chicken red blood cells were not satisfactory because they contain heterophile antigens as do many strains of P. multocida. Several samples of horse cells were also found to be unsatisfactory.

Sensitization of Erythrocytes with Extract

To each bacterial extract consisting of approximately 3.0 ml. there was added 0.2 ml. of packed red cells. After thorough mixing, the extracts and cells were placed in the incubator at 37°C.
for two hours. The red cells were then separated by centrifugation and washed once with 10.0 ml. of normal saline after which sufficient saline was added to give a one per cent suspension.

**Preparation of Immune Serums**

Two sera, 1001 and 1305, were prepared in cattle and supplied through the courtesy of the Connaught Medical Research Laboratories. Six additional bovine sera were also available but were not used routinely. The other sera were prepared in rabbits. Satisfactory rabbit sera were usually obtained after approximately 10 intravenous injections of formalin (0.25%) killed suspensions. The organisms constituting the killed antigens were derived from young blood agar cultures. These organisms were washed in saline then diluted in formalized saline until each millilitre contained approximately $10^9$ cells. It was customary to give the first antigen injection subcutaneously. If sufficient titer was not obtained after the series of killed antigen injections, live cultures were administered subcutaneously. The dosage began at 0.25 ml. of a young broth culture and in some instances was increased gradually to 20.0 ml. All immune sera were absorbed by the addition of 0.2 ml. of packed washed human cells to each 1.5 ml. of serum. After incubation for several hours, the cells were removed by centrifugation. If this absorption was not sufficient the procedure was repeated.

**Performance of Haemagglutination Test**

Twofold serial dilutions of the immune type sera were made in saline in 12 mm. diameter tubes. The dilutions employed routinely are listed in Table 9. 0.4 ml. of a 1% suspension of treated red
blood cells were added to the tubes containing 0.4 ml. of the serum dilutions. The two control tubes used were: (1) 0.4 ml. of 1% suspension of treated cells plus 0.4 ml. of normal saline, (2) 0.4 ml. of 1:5 dilution of the serum used plus 0.4 ml. of a 1% suspension of erythrocytes. After it was learned that a serum did not possess agglutinins for the human cells, the second control tube was omitted.

The racks of tubes were shaken vigorously then left at room temperature for approximately two hours at which time a reading was taken. The tubes were then placed in the refrigerator and a second reading was made the following morning. Positive reactions were occasionally seen only at the second reading. This slower agglutination was attributed to a smaller amount of capsular antigen adsorbed to the red cells. To facilitate reading the test, the tubes were held over a well lighted microscope mirror. A positive reaction consisted of marked agglutination while a negative test showed no evidence of clumping. The following morning the tests were read by shaking the tubes lightly to dislodge the cells. The cells were then allowed to settle and a reading was made over a mirror as described above.

**Strains of P. multocida Employed**

These are listed in Table 10 according to their animal origin and other data. A number of strains could not be typed initially because of the absence of the capsular antigen. The type specific capsular antigens of two of these strains, 1000(A) and 398(C) were restored by passage in mice. Noncapsulated variants are detectable
on transparent agar by their failure to show fluorescence. Freshly isolated strains are usually capsulated. Some avian strains however, are either noncapsulated or are only sparsely capsulated on primary isolation. One or two mouse passages were not always successful in restoring the capsular antigen.

**Absorption of Immune Serum with Bacteria**

To 1 ml. of serum to be absorbed there was added the conflu-ent growth from one blood agar plate. The organisms were removed from the plate with saline and collected as a centrifugate. The mixture of serum and bacteria was incubated for several hours after which separation was accomplished by centrifugation.
The results obtained with three cultures, the serological identity of which had been established by other methods, are presented in Table 9. Only cultures yielding a predominance of smooth, virulent variants were employed. The haemagglutination confirmed in a highly specific manner the serological identity of the strains. A fourth serological type identified as D by precipitation and capsular swelling tests was also demonstrated by the haemagglutination test.

The greater number of strains of *P. multocida* isolated at the Ontario Veterinary College have been of the mucoid variety. These cultures on primary isolation are composed for the most part of large moist colonies the organisms of which possess large capsules composed of hyaluronic acid. When these strains were examined by the haemagglutination test it was noted that haemagglutination to a high titer occurred with the bovine type sera but not with any of the immune rabbit sera.

To demonstrate the serological distinction between the mucoid substance and the specific antigen, type sera 1000(A) and 1305(B) were absorbed with the markedly mucoid strain 323 of swine origin. The type specific antibodies were not removed as both the absorbed sera agglutinated the red cells treated with the homologous type specific antigen. This experiment was repeated with the same results employing for the absorptions, two other mucoid strains.

In Table 10 the strains of *P. multocida* examined in this study are listed according to their animal origin and serological
The Type Specificity of Strains of Pasteurella multocida as Shown by the Haemagglutination Test

<table>
<thead>
<tr>
<th>Serum</th>
<th>Culture Extract</th>
<th>10</th>
<th>20</th>
<th>40</th>
<th>80</th>
<th>160</th>
<th>320</th>
</tr>
</thead>
<tbody>
<tr>
<td>1001(A)*</td>
<td>1001(A)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>1001(A)</td>
<td>1305(B)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1001(A)</td>
<td>361(C)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1305(B)</td>
<td>1001(A)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1305(B)</td>
<td>1305(B)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>1305(B)</td>
<td>361(C)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>361(C)</td>
<td>1001(A)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>361(C)</td>
<td>1305(B)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>361(C)</td>
<td>361(C)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

* Serological type as determined by the precipitation test.
<table>
<thead>
<tr>
<th>Types</th>
<th>Not Typing</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mucoid</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Swine</td>
<td>1</td>
</tr>
<tr>
<td>Cattle</td>
<td>3</td>
</tr>
<tr>
<td>Poultry</td>
<td>17</td>
</tr>
<tr>
<td>Buffalo</td>
<td>1</td>
</tr>
<tr>
<td>Rabbit</td>
<td>0</td>
</tr>
<tr>
<td>Sheep</td>
<td>0</td>
</tr>
<tr>
<td>Goat</td>
<td>0</td>
</tr>
<tr>
<td>Man</td>
<td>0</td>
</tr>
<tr>
<td>Dog</td>
<td>0</td>
</tr>
<tr>
<td>Cat</td>
<td>0</td>
</tr>
<tr>
<td>Deer</td>
<td>0</td>
</tr>
<tr>
<td>Mink</td>
<td>0</td>
</tr>
<tr>
<td>Chinchilla</td>
<td>0</td>
</tr>
<tr>
<td>Totals</td>
<td>22</td>
</tr>
</tbody>
</table>
Types A and B were generally associated with acute pasteurellosis in cattle and buffalo. Type A strains predominate as the cause of fowl cholera. Only type C strains have been recovered from cats and dogs. Type D infections appear to be generally sporadic and have a wide host range. Although one culture from atrophic rhinitis was identified as type B previously (23), all strains examined in this study from this disease have been either type D or mucoid. Noncapsulated cultures and mucoid strains as previously observed, were generally associated with carrier states and chronic processes.
A further study of type cultures, kindly supplied by Roberts, showed that his types I, II, III, and IV are identical respectively with types B, A, C, and D of this study.

Of particular interest is the observation that red cells treated with the capsular hyaluronic acid of mucoid organisms were clumped by all the bovine type sera but by none of the rabbit sera. Even when almost totally mucoid strains were injected repeatedly into rabbits, no "anti-mucoid" antibody was demonstrable by the haemagglutination test. It may be that the doses of hyaluronic acid were sufficiently large to produce an "immunological paralysis". This phenomenon has been observed (38) with type specific pneumococcus polysaccharides.

Many of the cultures examined appeared to contain fluorescent variants as well as mucoid variants although the latter usually predominated. Because the mucoid substance was usually present in an amount larger than the specific antigen, the specific reaction was blocked. Some preliminary work indicated that this blocking could be averted if the hyaluronic acid in the bacterial extract is depolymerized with hyaluronidase.

The information brought forward in this part of the studies and in the foregoing two parts will enable laboratories to produce bacterins and antisera for the prevention of pasteurellosis along more rational lines. At the present time little effort is being made to select the predominant serological types recovered from
Pasteurellosis in cattle and fowl for the preparation of bacterins and antisera for use in these species. A requisite for the preparation of highly immunogenic and protective antisera is a knowledge of the dissociation pattern of *P. multocida*. It is particularly important that one be able to recognize the prevalent and nonimmunogenic mucoid variants.
SUMMARY OF PARTS I, II, AND III.

I. It has been demonstrated by means of two precipitation techniques and a capsular swelling test that Pasteurella multocida possesses a type specific capsular antigen presumably polysaccharide in nature. The antigens employed in the precipitation tests were recovered from virulent cultures by two techniques. In the first the capsular antigen was released by organisms suspended in normal saline and in the second the specific antigen was recovered by an extraction method. Antisera were produced in rabbits and chickens by the inoculation of formalized culture suspensions. On the basis of the type specific capsular antigen, three distinct serological types of P. multocida have been identified.

II. Capsular polysaccharides possessing different chemical, physical, and biological properties have been isolated from the fluorescent, intermediate, mucoid variants of Pasteurella multocida. Data are presented relative to yields obtained, percentage composition of nitrogen, reducing substances, and phosphorus. Hyaluronic acid was identified in the capsular substance from mucoid variants.

The fluorescent and intermediate soluble substances were highly active and specific serologically. The mucoid substance elicited little or no immunity in mice but the fluorescent substance was markedly immunogenic. Both substances were nontoxic for mice in doses up to 3 mg.
III. A haemagglutination test has been developed for the identification of serological types of *Pasteurella multocida*. This technique yields highly specific reactions and is considered to have advantages over other typing methods for this organism. The specific capsular antigens and hyaluronic acid capsular material were extracted with saline and adsorbed to human type O erythrocytes. To these treated cells were added appropriate dilutions of the specific typing sera. Positive reactions consisted of agglutination of red cells. One hundred and fifty-two strains of *P. multocida* from various geographical sources and animal species were typed by the haemagglutination test. Four different serological types, A, B, C, and D were recognized. A large number of mucoid strains and noncapsulated cultures which were not typable were also observed. The serological results are discussed briefly in relation to the nature and occurrence of pasteurellosis in the various species.
ACKNOWLEDGMENTS

The author is indebted to Dr. E. Annau of the Animal Diseases Research Institute, Hull, Quebec for carrying out the chemical determinations referred to in Part II.
REFERENCES

1. Aitken, W.
   1940 So-called Hemorrhagic Septicemia.

2. Alexander, Mary M., Wright, George G. and Baldwin, Agnes C.
   1950 Observations on the Agglutination of Polysaccharide-Treated Erythrocytes by Tularemia Antisera.
   J. Exp. Med. 91: 561-566.

   1929 Dissociation of the Bacillus avisepticus. Parts I and II.

4. Bain, R. V. S.
   1954 Vaccination Against Hemorrhagic Septicemia of Bovines.

5. Barnum, D. A.
   1954 A Herd Outbreak of Mastitis Caused by Pasteurella multocida.

6. Boyden, S. V.
   1951 The Adsorption of Protein on Erythrocytes Treated with Tannic Acid and Subsequent Haemagglutination by Anti-protein Sera.
   J. Exp. Med. 93: 107-120.

7. Breed, R. S., Murray, E. C. D. and Hitchens, A. P.
8. Burger, M.

1950 Bacterial Polysaccharides.  

9. Carter, G. R.

1951 Studies on a Pasteurellla multocida Chicken Embryo Vaccine II. Type Specific Nature of Immunity Elicited by a Monovalent Pasteurellla multocida Vaccine.  

10. Carter, G. R. and Byrne, J. L.

1953 A Serological Study of the Hemorrhagic Septicemia Pasteurellla.  


1953 Dissociation and Virulence in Strains of Pasteurellla multocida Isolated from a Variety of Lesions.  

12. Carter, G. R.

1954 Observations on the Pathology and Bacteriology of Shipp- 

ing Fever in Canada.  

13. Carter, G. R. and McSherry, B. J.

1955 Further Observations on Shipp- 

ing Fever in Canada.  

14. Carter, G. R.

1955 Virus Pneumonia of Pigs: A Discussion for Practitioners.  

15. Cornelius, J. T.

1929 An Investigation of the Serological Relationships of 26  
Strains of Pasteurellla.  
16. DeKruif, P. H.


17. Dingle, J. H.

1934 The Serological Specificity of Bacterial Carbohydrates, with Special Reference to Type II Pneumococcus and a Heterophile Strain of *Bacterium lepisesticum*. Amer. J. Hygiene 20: 148-168.


19. Fahey, J. E.


20. Gaiger, S. H.


21. Gochenour, Wm. S.


22. Glässer.

23. Gwatkin, R., Dzenis, L. and Byrne, J. L.

1953 Rhinitis of Swine VII. Production of Lesions in Pigs and Rabbits with a Pure Culture of Pasteurella multocida.

24. Gwatkin, R.

1955 A Discussion on Atrophic Rhinitis of Swine with Special Reference to Recent Work in Canada.

25. Hoffenreich, F.

1923 Kapselsubstanz aus Bacillus septicus.

26. Hughes, T. P.

1930 The Epidemiology of Fowl Cholera II. Biological Properties of Pasteurella avicida.

27. Jones, F.

1921 A Study of Bacillus bovisenticus.

28. Jorgensen, G. E.

1925 Some Studies of Pasteurella bovisenticus.

29. Kass, E. H. and Seastone, C. V.

1944 The Role of the Mucoid Polysaccharide (Hyaluronic Acid) in the Virulence of Group A Hemolytic Streptococci.

30. Kendall, F. E., Heidelberger, M., and Dawson, M. H. A.

1937 Serologically Inactive Polysaccharide Elaborated by Mucoid Strains of Group A Hemolytic Streptococcus.
   1948 Adsorption of Bacterial Polysaccharides to Erythrocytes.

32. Khalifa, I. A. B.
   1936 Pasteurellae in Animals and Their Classification.
   (Abstr. in Vet. Bull. 6: 792 (1936).)

33. Landy, M.
   1954 On Haemagglutination Procedures Utilizing Isolated Polysaccharide and Protein Antigens.

34. Lignières, J. M.
   1901 Contribution a l'étude et a la classification de septi- 
   cémies hémorragiques. Les Pasteurelloses.

35. Little, F. A. and Lyon, B. M.
   1943 Demonstration of Serological Types within the Nonhemolytic Pasteurella.

36. MacPherson, C. F. C.
   1943 A Method of Typing Haemophilus influenzae by the Precipitin Reaction.

37. Migge.
   1933 Das Virus der Wild- und Rinderseuche.

   1952 Immunization of Rabbits with Type II Pneumococcus Polysaccharide.
39. Newson, I. E. and Cross, F.
1932 Some Bipolar Organisms Found in Pneumonia in Sheep.

40. Ochi, Y.
1931 Studies on Hemorrhagic Septicemia, Especially on Their
1953 Variability, 4 Reports.
and 12: 47-52 respectively.

41. Olsen, A. M. and Needham, G. M.
1952 Pasteurella multocida in Suppurative Diseases of the
Respiratory Tract.

42. Pirosky, I.
1938 Sur l'antigène glucido - lipidique des Pasteurella.

43. Pirosky, I.
1938 Sur les propriétés immunisantes antitoxiques et anti-
infectieuses de l'antigène glucido - lipidique de Pas-
teurella avisentica.

44. Pirosky, I.
1938 Sur l'existence, chez les variants smooth et rough d'une
souche de Pasteurella avisentica de deux antigènes
glucido - lipidiques serologiquement distincts.

45. Pittman, M.
1931 Variation and Type Specificity in the Bacterial Species
Hemophilus influenzae.
46. Priestley, F. W.

1936 Experiments on Immunization Against Pasteurella septica Infection.

47. Pritchett, I. W., Beaudette, F. R., Hughes, T. P.

1930 The Epidemiology of Fowl Cholera IV. Field Observations of the Spontaneous Disease.

48. Pritchett, I. W., Beaudette, F. R., Hughes, T. P.

1930 The Epidemiology of Fowl Cholera V. Further Field Observations of the Spontaneous Disease.
J. Exp. Med. 51: 259-274.

49. Rao, S. B. V.

Personal Communication.

50. Rice, C. E., Beauregard, M. and Maybee, T. K.

1955 Survey of Shipping Fever in Canada: Serological Studies.

51. Roberts, R. S.

1947 An Immunological Study of Pasteurella septica.
J. Comp. Path. 57: 261-273.

52. Roderick, L. M.

1922 Some Antigenic Relations of the Bipolaris septicus Group of Bacteria.

53. Rosenbusch, C. T. and Merchant, I. A.

1939 A Study of the Hemorrhagic Septicemia Pasteurellae.
54. Seastone, C. V.
1939 The Virulence of Group C Hemolytic Streptococci of Animal Origin.

55. Smith, J. E.
1955 Studies on Pasteurella septica I. The Occurrence in the Nose and Tonsils of Dogs.
J. Comp. Path. 65: 239-245.

56. Tweed, W.
1932 Hemorrhagic Septicemia in Bovines.

57. Webster, L. T. and Burn, C. G.
1926 Biology of Bacterium lepisencicum III. Physical, Cultural, and Growth Characteristics of Diffuse and Mucoid Types and Their Variants.

58. Webster, L. T. and Burn, C. G.
1926 Biology of Bacterium lepisencicum IV. Virulence of Diffuse and Mucoid Types and Their Variants.

59. Yusef, H. S.
1935 Serological Classification of Pasteurella Strains.

60. Zaisen, K.
1934 Serological Studies on Hemorrhagic Septicemia Organisms, Especially on Their Complement-Fixation Reaction.
<table>
<thead>
<tr>
<th>DATE DUE</th>
</tr>
</thead>
<tbody>
<tr>
<td>JAN 21, 1957</td>
</tr>
<tr>
<td>FEB 7, 1957</td>
</tr>
<tr>
<td>MAY 2, 1957</td>
</tr>
<tr>
<td>APR 25, 1958</td>
</tr>
<tr>
<td>MAY 28, 1958</td>
</tr>
<tr>
<td>AUG 24, 1958</td>
</tr>
<tr>
<td>OCT 2, 1958</td>
</tr>
<tr>
<td>FEB 16, 1960</td>
</tr>
<tr>
<td>MAR 3, 1964</td>
</tr>
<tr>
<td>FEB 3, 1965</td>
</tr>
<tr>
<td>DEC 29, 1969</td>
</tr>
<tr>
<td>NOV 30, 1970</td>
</tr>
<tr>
<td>DEC 21, 1970</td>
</tr>
</tbody>
</table>
THESIS:
SF 756.T
C 245
1956
Carter, Gordon R.
A serological classification of pasteurelia multocida.