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A COMPARATIVE STUDY OF MAMMALIAN AND AVIAN ISOLATES OF *PASTEURELLA HAEMOLYTICA*

By

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ABSTRACT

A COMPARATIVE STUDY OF MAMMALIAN AND AVIAN ISOLATES OF *PASTEURELLA HAEMOLYTICA*

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This investigation was carried out to characterize avian isolates of *P. haemolytica* according to the following characteristics: 1) by fermentation studies, 2) by growth studies, 3) by pathogenic studies, 4) by electron microscopic studies, 5) by serological studies, and 6) by guanine-cytosine ratio estimation. The fermentation studies revealed that the avian strains may constitute a distinct biotype or new species, as there were considerable fermentative variations from those of mammalian strains of *P. haemolytica*. The growth studies indicated that the avian strain had a lag phase of 1.5 hours as compared to 3 hours for the mammalian strain, and also the former multiplied logarithmically for 1.5 to 6 hours, whereas the latter multiplied logarithmically from a period of 3 to 10 hours.

A 2% hemoglobin preparation was found to be a nontoxic, better enhancer of virulence than 7% swine gastric mucin. The mammalian strains were found to be more virulent for mice than the avian strains, but the difference was not statistically significant ($p>0.05$). The pathogenicity of avian isolates to White Leghorn birds depended on the age of the bird, the size of the inoculum and the route of inoculation. Electron microscopic studies on thin sections revealed that the cell

membrane of avian cultures was tightly attached to the cell and the membrane was ca. 62 Å thicker than that of mammalian strains.

The counterimmunoelectrophoresis (CIE) test was used successfully to identify all 12 serotypes of *P. haemolytica*. This test was found to be superior to the rapid plate agglutination and the indirect hemagglutination tests. Fifty-seven of the avian cultures were grouped into 6 distinct capsular types by CIE and they were designated numerically 1 through 6. The guanine-cytosine ratios in the DNA samples of *Pasteurella* and *Actinobacillus* species were estimated by 2 different methods. The data indicated that these DNAs were genetically closely related. The results of present investigation strongly suggested that the avian cultures should be retained in the genus *Pasteurella* with a new species name, viz., *Pasteurella avihaemolytica*.

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INTRODUCTION

Pasteurella haemolytica is a gram-negative pleomorphic nonmotile rod characterized by a fermentative carbohydrate metabolism. Concerning oxygen requirements, *P. haemolytica* is aerobic and facultatively anaerobic. The temperature range for growth is 22 to 42 C with 37 C being optimal.

The occurrence and significance of *P. haemolytica* as a potential pathogen has only received wide recognition in recent years. Although it is commonly isolated from the nasal passages of healthy animals and of animals with respiratory diseases, its role in respiratory disease is not well defined (Carter, 1967; Collier, 1968a,b; Lillie, 1974). Epidemiological studies have shown that the number of calves carrying *Pasteurella* in their nasopharynx increases during outbreaks of shipping fever (Hoerlein et al., 1961) and in 2 weeks after transportation (Thomson et al., 1969). Under certain stress or disease conditions, the *P. haemolytica* can replicate rapidly and can reach the alveoli in aerosolized droplets. The healthy host will clear the bacteria rapidly from lungs, but the diseased or stressed host may develop pneumonia.

Biberstein et al. (1960) divided isolates from cattle and sheep into 12 serotypes by an indirect hemagglutination (IHA) procedure. Smith (1959 and 1961) divided the species into biotypes A and T mainly on the basis of colonial morphology and fermentation of arabinose and trehalose. Later, when the 12 serotypes were grouped according to

biotype, serotypes 3, 4 and 10 were biotype T and all others were biotype A. Only a limited number of complete serotyping studies have been done on large numbers of *P. haemolytica* isolates owing to the time and effort involved in the IHA procedure. Thus far, serotype 1 is the predominant serotype isolated from cattle; however, serotype 2 and untypable isolates are also frequently isolated (Biberstein et al., 1960; Fox et al., 1971; Frank and Wessman, 1978; Mwangota et al., 1978). Serotypes 3, 4, 6, 7, 9 and 11 have been infrequently isolated from cattle (Frank and Wessman, 1978; Mwangota et al., 1978; Wessman and Hilker, 1968).

Organisms described as *P. haemolytica* in the literature but derived from other hosts, including horses, swine, chickens and humans have only, in exceptional cases, been typable serologically. These isolates differ in biochemical and cultural characteristics from those of cattle and sheep (Heddleston, 1975; Biberstein et al., 1960; Biberstein, 1978). Their assignment to the species is often questionable. This study was carried out to (1) develop a more sensitive mouse model to evaluate the virulence of *P. haemolytica*, (2) develop a more simple, fast and accurate serotyping technique for *P. haemolytica*, and (3) regroup avian isolates of *P. haemolytica* into a new variety or possibly a new species under the same genus *Pasteurella*. To accomplish this, the study was carried out in the following areas: virulence of the organisms to mice and chickens, ultrastructural analysis by electron microscopy, serologic typing by using the counter-immunoelectrophoresis technique, biochemical reactions, growth studies and guanine-cytosine ratio estimation of the organisms.

REVIEW OF LITERATURE

Systematics

Bacteria identical to those referred to as *Bacillus bovisepticus* Group 1 by Jones (1921) were given the name *Pasteurella haemolytica* by Newsom and Cross (1932). However, some workers (Lovell and Hughes, 1935; Bosworth and Lovell, 1944) were not prepared to classify *B. bovisepticus* in the genus *Pasteurella* and preferred to use the name hemolytic coccobacilli. Marsh (1932) recovered strains from mastitis in ewes and named them *Pasteurella mastitidis*. An earlier examination of strains of *P. mastitidis* from Montana revealed that the cultural and biochemical characteristics were indistinguishable from those of typical strains of *P. haemolytica* (Carter, 1951).

By current standards (Smith, 1974; Phillips, 1974), *Actinobacillus* possesses beta-galactosidase and urease activity. *Pasteurella* species may have one or the other enzyme, rarely both. The suggestion has been made (Bohácek and Mráz, 1967) that *P. haemolytica* is more closely related to the genus *Actinobacillus*, as it is the only species of *Pasteurella* capable of some growth on MacConkey agar and harboring subtypes with beta-galactosidase capability. Mráz (1969) estimated the guanine-cytosine content and suggested that *P. haemolytica* be excluded from the genus *Pasteurella* and transferred to the genus *Actinobacillus*.

The diagnostically important characteristics of the species of *Pasteurella* and the genus *Actinobacillus* are summarized in Table 1 (Biberstein, 1978).

Table 1. Differential characteristics of *Pasteurella* species and the genus *Actinobacillus*

	Hemolysis	Indole	Urease	ONPG	Growth on MacConkey agar
Pasteurella multocida	-	+	-	-	-
Pasteurella pneumotropica	-	+	+	a	-
Pasteurella ureae	a	-	+	-	-
Pasteurella haemolytica	+	-	-	a	+
Actinobacillus spp.	a	-	+	+	+

[a]Different reactions possible.

Cultural and Biochemical Characteristics

Morphologic Features

Species of the genus *Pasteurella* are gram-negative coccobacilli measuring 1.4 ± 0.4 by 0.4 ± 0.1 µm. Organisms are capsulated especially in fresh isolates. Long filamentous forms are frequently seen in old laboratory cultures. In the Gram's-stained smear, *P. haemolytica* can exhibit bipolar staining on initial isolation. Strains of *P. haemolytica* are morphologically indistinguishable from *Pasteurella multocida*.

On blood agar at 37 C, 24-hour colonies are 0.5 to 1 mm in diameter, smooth, but often with concentric rings, entire and translucent; older colonies are larger. A zone of hemolysis surrounds

colonies of freshly isolated strains but may be reduced or lost after a few subcultures (Smith, 1974). However, strains isolated from avian species possess a wider zone of hemolysis (Harbourne, 1962; Janetschke and Risk, 1970).

Variation

Biberstein et al. (1958) observed that primary cultures of *P. haemolytica* from 3 lambs were made up of 2 different colonial variants, which he designated S and R on the basis of their behavior in the crystal violet "uptake" test. Some characteristics of these variants are summarized in Table 2. Wessman (1964) studied the interrelationships

Table 2. Differential characteristics of S and R variants of *P. haemolytica*

S Variant	R Variant
Dome shaped and grayish white	Conical and faintly gray
Did not take up crystal violet	Took up crystal violet
Adherent to agar after dye application	Nonadherent
Pathogenic for lambs	Pathogenic for lambs
Pathogenic for mice	Less pathogenic for mice
Agglutinable by S serum	Agglutinable by S and R sera
Abundant surface substance	Deficient in surface substance

of smooth and nonsmooth cells in the dissociation of *P. haemolytica* grown in broth. The critical factor in the inhibition of the growth of smooth cells appeared to be limitation of oxygen in the medium. Selective inhibition was not observed in the aerated cultures.

Biochemical Activity

Considerable variation has been recognized in the fermentative activity of strains of *P. haemolytica*. Smith (1961) observed that all 14 of his type A strains fermented arabinose in 7 days, while 14 type T strains did not. He also found with the same strains that the type T cultures fermented trehalose in 10 days, while the type A strains did not. Only 3 of the 28 isolates split lactose and these belonged to type A.

Biberstein et al. (1960) reported that their capsular type 1 strains were, with several exceptions, lactose and catalase positive, while all of their type 2 strains were lactose negative and catalase positive. All of their type 3 cultures were lactose and catalase negative. These characteristics alone were not sufficient to categorize strains, in that the same combinations were found with other capsular types.

Shreeve et al. (1970) reported that the use of the fermentation reactions with arabinose and trehalose to distinguish the A and T biotypes was found to be more reliable for T than for A strains, since some strains of biotype A in all serotypes except serotype 11 failed to ferment arabinose within 14 days. Isolates of *P. haemolytica* from chickens and turkeys were found to be different in their fermentative activity from the strains isolated from cattle and sheep (Heddleston, 1975). Similar findings were reported by Janetschke and Risk (1970), who observed considerable biochemical variation among the isolates of avian origin.

The *Pasteurella* species can be differentiated on the basis of the reactions presented in Table 3 (Carter, 1967, 1979; Smith, 1974).

Table 3. Differentiation of *Pasteurella* species

	Mac-Conkey	Hemo-lysis	Indol	Urea	Glucose	Lactose	Mannitol
P. multocida	-	-	+	-	A	N	(A)
P. haemolytica	+	β	-	-	A	(A)	A
P. ureae	-	α	-	+	A	N	A
P. pneumotropica	-	-	+	+	A	(A)	N
P. gallinarum	-	-	-	-	A	N	N
P. aerogenes	+		-	+	A (gas)	N	N
P. anatipestifer	-	-	-	-	N	N	N

A = fermentation; N = no fermentation; (A) = most ferment

Nutrition and Cultivation

Berkman (1942) studied the nutritional requirements of various species of *Pasteurella* and reported that the isolates of *P. haemolytica* grew well in a medium containing hydrolyzed gelatin, amino acids, glucose and inorganic salts. Wessman (1965) studied the growth pattern of *P. haemolytica* strain H44L under aerobic conditions in a medium containing acid-hydrolyzed casein, cysteine, inorganic salts, vitamins and carbon source. The best carbon source was found to be D-galactose or sucrose. From the same study, Wessman observed that the maximal growth resulted from an inoculum containing fewer than 10 cells per milliliter of medium. A year later, a chemically defined medium was developed by Wessman (1966) to cultivate *P. haemolytica* under aerobic conditions.

Wessman and Wessman (1970) studied the thiamine requirement of *P. haemolytica* and found that 2 of 11 isolates of *P. haemolytica* grew well with normal amounts of thiamine; the remaining 9 isolates could satisfy their thiamine requirement with free thiamine only if large amounts were provided.

Typing

History of Typing

Two general approaches to typing of *P. haemolytica* have been taken. One, developed by G. R. Smith (1959, 1961), has resulted in the division of the species into 2 types, A and T. The letters stand for arabinose and trehalose fermentation, respectively, and this fermentation characteristic of the members of the respective types. The other approach is serological and, in its present form, was developed by Carter (1956) and Biberstein and co-workers (1960).

Prior to the emergence of the present methods, attempts at subdividing the species have been more sporadic. Serological studies prior to 1960 by agglutination (Florent and Godbille, 1950; Montgomerie et al., 1938; Newsom and Cross, 1932; Tweed and Edington, 1930) and hemagglutination (Carter, 1956) suggested the existence of no more than 3 types. One of these was undoubtedly the present type 1, which predominates in bovine infections.

It is difficult to assign the cultures described in these older reports to one of the 2 biotypes A and T, as the data are not completely consistent with either. In all likelihood the overwhelming majority were of type A. Only 2 cultures studied by Newsom and Cross (1932), the proponents of the species name, appear at all compatible with type T criteria.

In 1959 and 1961, Smith reported the occurrence of 2 types of *P. haemolytica* in sheep, distinguishable by a number of cultural traits. Moreover, there were pathogenic and epidemiologic differences between the 2 types, which Smith designated A and T, after their reactivity in arabinose and trehalose broth, as previously noted.

A serological study by Carter (1956) of 51 bovine isolates failed to disclose any type of diversity by IHA and agglutination procedures. Subsequently, an investigation employing similar methods on 98 isolates of *P. haemolytica* from sheep and cattle and some other species revealed the existence of 10 capsular types, some of which showed some distinct biochemical, ecologic, and pathogenic patterns (Biberstein et al., 1960). In 1962, an investigation (Biberstein and Gills, 1962) into the relationship of the A and T "biotypes" to the serotypes disclosed, in the sample studied, a consistent association between serotype and biotypes (Table 4). Subsequently, 2 additional serotypes were identified (Biberstein and Gills, 1962; Biberstein and Thompson, 1966), so that at present 12 serological types are recognized.

Biotyping

G. R. Smith (1959, 1961) found that strains of *P. haemolytica* from ovine pneumonia could be separated into 2 major groups on the basis of different cultural, biochemical, ecological, and pathologic characteristics. The differences were not attributable to colonial variation, as was the case with the S and R variants of Biberstein et al. (1958). Smith's groups were designated types A and T. The major distinguishing features are summarized in Table 4.

Table 4. Differential characteristics of subtypes of *P. haemolytica* (Smith, 1974)

Biotype	A	T
Fermentation of arabinose	+	-
trehalose	-	+
salicin	-	+
xylose	+[a]	-
lactose	d	-
Susceptibility to penicillin	high	low
Serotypes	1,2,5,6,7,8,9,11,12	3,4,10
Principal localization in normal host	nasopharynx	tonsils
Principal disease association	pneumonia of cattle and sheep; septicemia of nursing lambs	septicemia of feeder lambs

[a] Serotype 2 negative, all others positive

Biotype T colonies tend to be slightly larger, measuring up to 2 mm in diameter, and possess large, dark, brownish centers, whereas biotype A colonies have an even grayish color and sometimes a small, demarcated central thickening (Smith, 1961).

Smith (1961) used the bromthymol blue medium of Bosworth and Lovell (1944) to study the fermentation reaction of A and T biotypes. Subsequent workers have substituted bromcresol purple broth (Biberstein and Gills, 1962) or phenol red broth (Wessman and Hilker, 1968). The most useful substrates by most accounts are arabinose, trehalose, lactose and salicin. Arabinose is fermented only by type A strains, trehalose only by type T. Lactose is not fermented by type T strains, while salicin is usually attacked by type T, infrequently by type A. Mannose

has been reported to be more frequently fermented by T than A strains, while the opposite has been described for xylose (Fredriksen, 1973; Shreeve et al., 1970).

Smith (1961) reported the difference of growth and death patterns between types A and T. He established, on the basis of testing 31 different isolates, that growth curves of A and T strains were essentially indistinguishable at the initial stage of incubation, but subsequent incubations maintained a higher T strain population.

Most of the data on antimicrobial sensitivity patterns between A and T types are of a qualitative nature. Smith (1961) observed that the type A strains were more sensitive to penicillin than type T strains. Olmos and Biberstein (1979) described a method to differentiate A and T strains of *P. haemolytica* using growth inhibitors. They found that the paper disks containing 0.3 µg of penicillin-G produced zones of inhibition larger than 10 mm with type A but not with type T strains. Also, basic fuchsin (0.2 µg/ml), brilliant green (0.005 µg/ml), and methylene blue (3.1 µg/ml) in brain-heart infusion broth permitted the growth of type T strains but not type A.

Serotyping

Present-day serotyping of *P. haemolytica* is concerned entirely with soluble, presumably surface, antigens. These antigens have been identified as polysaccharide (Cameron, 1972) or lipopolysaccharide (Carter, 1967). The IHA procedure in one of its several modifications is usually employed to detect the antigens (Biberstein et al., 1960; Biberstein and Thompson, 1966; Biberstein et al., 1970). Recently, a rapid plate agglutination (RPA) test has been developed to detect the same surface antigens of *P. haemolytica* (Frank and Wessman, 1978).

Carter (1956) observed that 51 isolates of *P. haemolytica* from pneumonic lungs of cattle were found to be serologically homogeneous by an IHA test. Because all the 51 isolates were found to be of one type, Carter (1956) mistakenly assumed a widespread homogeneity of pathogenic strains. Biberstein et al. (1960) examined 98 strains of *P. haemolytica* by means of a modified IHA test. Ten types were identified on the basis of differences in capsular substances and 18 isolates were not typable. Types were designated by arabic numbers 1 through 10. Subsequently, 2 additional serotypes (11 and 12) were identified by IHA test (Biberstein and Gills, 1962; Biberstein and Thompson, 1966). More recently, 2 new serotypes (13 and 14) were identified from sheep (Pegram et al., 1979), but their official recognition is yet to be realized.

Frank and Wessman (1978) developed a simple RPA test to type *P. haemolytica*, based on surface antigens. Of the 103 isolates, 95 were of the same serotype, as determined by both RPA and IHA tests, and 5 were untypable. The remaining 3 isolates were typable with the RPA test but not with IHA. Biberstein et al. (1960) examined 98 isolates of *P. haemolytica* by an agglutination procedure employing autoclaved bacteria and the same sera used for the IHA tests. Thirteen somatic groups were identified by this test and designated A, AB, AD, B, BD, C, D, E, F, G, H, I and V. The division into somatic groups on the basis of agglutination was not so clearcut as the division into types based on IHA. There was no reciprocity of reactions, suggesting the presence of haptens rather than complete antigens in the somatic portions.

Muraschi et al. (1965), employing ether-extracted antigens by the method of Ribi et al. (1959), were able to type strains of *P. haemolytica* with a modification of the Ouchterlony gel diffusion technique.

Problems in Typing

Determination of biotypes may not always be clearcut and straightforward. Differences in colonial morphology are subtle and are identified with greatest realiability when large colonies of the 2 biotypes occur side by side. Biberstein et al. (1958) observed that colonial dissociation within the strain occurred at varying frequencies and obscured the differences further. Variable arabinose fermentation reactions have been reported for biotype A by Carter (1976), Cowan (1974) and Frederikson (1973). The variability of fermentation tests, according to the medium employed, was stressed by Wessman and Hilker (1968). Problems were encountered in biotyping *P. haemolytica* isolated from poultry, as their fermentation reactions were different from those of cattle and sheep isolates (Heddleston, 1975).

The main limitation of serotyping is that not all isolates identifiable bacteriologically as *P. haemolytica* can be assigned to one of the 12 serotypes. In most cases the problem was found to be due, not to their belonging to an unrecognized type, but to their lack of the surface antigen which forms the basis for typing (Biberstein, 1978). Although cross reactions have been reported between serotypes, in most cases it was possible to eliminate the problem by using the type sera at higher dilution (Biberstein, 1965). Heddleston (1975) reported that none of the 20 isolates from poultry reacted with sera prepared against 12 types of *P. haemolytica*. Similar problems have been encountered in the past with avian and equine isolates of *P. haemolytica* (Biberstein et al., 1960). Although Frank (1980) was able to identify 3 serotypes among untypable bovine isolates by the RPA test, the problem remains to be solved with avian and equine isolates.

Relation of Biotypes and Serotypes

After the description of the A and T biotypes was published, a study was instituted to determine the distribution of these biotypes among representative strains of each of the 11 serotypes known at the time. The investigation by Biberstein and Gills (1962) revealed that all strains of types 1, 2, 5 through 9 and 11 were of type A, while all strains of types 3, 4 and 10 were of type T (Table 4). When type 12 was identified, it was found to belong to biotype A (Table 4) (Biberstein and Thompson, 1966). These relations between serotypes and biotypes were fully confirmed by a later investigation by Shreeve et al. (1970). Mwangota et al. (1978) reported the occurrence of both A and T types within each of the serotypes 3, 4, 6, 10 and 12, which conflicts directly with the previous experience. The conflicting results of Mwangota were believed to be due to high frequency of the unexpected biotypes of *P. haemolytica* (Biberstein, 1978). The 2 new serotypes (13 and 14) identified in Ethiopia were recognized as biotype A (Pegram et al., 1979).

Distribution and Prevalence of Types

Geographical

Both biotypes and all 12 serotypes have been identified wherever *P. haemolytica* has been studied extensively, viz., Great Britain (Biberstein and Thompson, 1966; Thompson et al., 1977), Kenya (Mwangota et al., 1978), Ethiopia (Pegram et al., 1979) and the United States (Carter, 1956; Biberstein et al., 1960; Wessman and Hilker, 1968). In the Republic of South Africa (Cameron, 1972), 50 isolates from pneumonic sheep were found to represent 10 serotypes, all except types

3 and 11. *Pasteurella haemolytica* has been isolated from pneumonic lungs of Australian sheep kept in quarantine at the port of San Diego. The serological identity of these isolates was determined by standard IHA test (Biberstein, 1978).

Zoological and Anatomical

Smith (1959), in his original description of the A and T types, emphasized that all of his T types were derived from cases of septicemia in feeder lambs (3-12 months old), while all the A types were obtained from pneumonic sheep. Subsequently, Smith (1961) reported septicemia in infant lambs (less than 3 months old) to be associated with type A. Biberstein and Thompson (1966) suggested that type T strains, being (1) less frequent on the whole, (2) extremely rare in the normal nasopharynx, (3) usually associated with clinical infections, and (4) alone capable of causing septicemic pasteurellosis in older lambs, were of greater pathogenic potential and lesser adaptation to a commensal existence.

Type A has been found in a high proportion of cases of respiratory disease, including shipping fever (Wessman and Hilker, 1968), and found frequently as part of the apparently normal nasopharyngeal population (Magwood et al., 1969). Tonsillar infection by *P. haemolytica* in 50 clinically normal adult sheep revealed a 3:1 preponderance of T over A strains in contrast to the usual biotype distribution in the nasopharynx of the same animal (Gilmour et al., 1974).

Within biotype A, it is serotype 1 which is most often encountered and apparently the only one associated with epidemic respiratory disease (Wessman and Hilker, 1968). Of the remaining types, 7 and 11 have been identified in cattle, while all of the 3 serotypes associated

with biotype T (3, 4 and 10) have been infrequently reported in that host (McDonald, 1974). The relationship of serologic type to animal origin and disease, as compiled by Smith (1974), is summarized in Table 4.

The report of Mwangota et al. (1978) revealed that the occurrence of *P. haemolytica* in goats is much like that in sheep, with all serotypes present but type 11 clearly in the lead.

Hemolytic, fermentative, oxidase-positive, gram-negative coccobacilli have been isolated from swine (Biberstein et al., 1960), horses (Guerrero et al., 1973), and poultry (Harbourne, 1962; Janetschke and Risk, 1970). Although these isolates were identifiable bacteriologically as *P. haemolytica*, the serologic identity remained unknown.

Antigenic Nature

The antigenic nature of *P. haemolytica* has received little attention. Kress et al. (1964) extracted endotoxin from a bovine strain. The product was evaluated for its dermotoxic effect in rabbits and its hemodynamic effect in sheep but was not studied serologically or immunologically. In its general pharmacologic effects, it resembled the endotoxins of other gram-negative bacteria. Adamou et al. (1972) characterized the proteins of *P. haemolytica* and *P. multocida* by vertical polyacrylamide gel disc electrophoresis. They observed that the differentiation of cultures was possible on the basis of different combinations of distinct bands of proteins with an acid isoelectric point. There was a distinct common protein band in all the cultures of *P. haemolytica* and *P. multocida* studied by this method. Three years later, Thompson and Mould (1975) studied the protein electrophoretic patterns of 12 serotypes of *P. haemolytica* employing polyacrylamide gel

electrophoresis. Although they were able to separate the biotypes A and T, many serotypes showed only a minor or no difference in their protein electrophoretic mobility patterns.

Vaccination and Immunity

Bacterins containing heavy concentrations of *P. multocida* and *P. haemolytica* were employed for the prevention of shipping fever in cattle with limited success (Carter, 1970). The work of Carter (1956) and Biberstein et al. (1960) indicated that type 1 strains should be included for the prevention of *P. haemolytica* infections in cattle. Hamdy and Trapp (1964) showed, in vaccination and challenge experiments, that immunity was obtained as a result of vaccination with a preparation containing parainfluenza-3 virus and strains of *P. multocida* and *P. haemolytica*. In subsequent experiments, Hamdy and associates (1965) found that the same preparations gave no protection against shipping fever in the field.

Biberstein and Thompson (1965) demonstrated the importance of serotype in determining the specificity of protection of mice by using bacterins. Subsequently, it was shown that bacterins made from cultures having no demonstrable serological relationship to the eventual infecting strain could protect mice effectively against this strain, better, in fact, than homologous bacterins (Knight et al., 1969).

Studies have been conducted on antibody response to *P. haemolytica* antigens. Calves in normal herds were found to have low antibody titers to several serotypes of *P. haemolytica* by the IHA procedure (Wray and Thompson, 1973). Intravenous and subcutaneous injections of live or killed *P. haemolytica* resulted in a nonsignificant serum antibody rise and no nasal washing antibody response (Duncan and Thomson,

1970a). From this same study, Duncan and Thomson found that the aerosol exposures with live *P. haemolytica* caused both serum antibody and nasal washing antibody responses, while aerosol exposure with heat-killed *P. haemolytica* resulted in lower serum antibody titers and no nasal washing antibody response. Some calves with nasal washing antibody titers to *P. haemolytica* serotype 1 were found to harbor serotype 1 in their nasal passages (Duncan and Thomson, 1970b).

Several basic problems make testing the prophylactic value of *P. haemolytica* vaccines difficult. *Pasteurella* pneumonia cannot be produced under experimental conditions with reproducible regularity. Attempts have been made over the years to develop an effective immunogen for the prevention of *P. haemolytica* infection in cattle and sheep, but the basic problems remain unsolved (Cameron, 1972; Matsuoka et al., 1972; Wilkie and Norris, 1976; Lopez et al., 1976). Recently, Wilkie and associates (1980) observed the adverse response to challenge exposure with *P. haemolytica* in subcutaneously vaccinated calves. Although the evidence was not clear for the adverse response, based on *in vitro* study it was found to be due to increased bacterial-induced macrophage cytotoxicity.

MATERIALS AND METHODS

I. Fermentation Studies

Cultures

These included 49 isolates from chickens, 9 from turkeys, and 1 from a parakeet. The isolates were identified as *P. haemolytica* by the characteristics as described by Carter (1979) and Smith (1974). The source and origin of the isolates are listed in Table 5.

Table 5. Isolates of *P. haemolytica* listed by source and origin

Number of isolates	Origin	Source
2	Chicken lung	National Animal Disease Center, Ames, IA
21	Chicken lung	VDL[a], MSU[b], East Lansing, MI
26	Chicken intestine	VDL, MSU, East Lansing, MI
9	Turkey lung	Animal Sciences Department, MSU, East Lansing, MI
1	Parakeet lung	VDL, MSU, East Lansing, MI

[a]VDL = Veterinary Diagnostic Laboratory

[b]MSU = Michigan State University

Fermentation Tests

Each culture was inoculated into tubes containing 1% carbohydrate substrate and 0.15% agar in phenol red broth base (Difco). The substrates used in the study were glucose, lactose, sucrose, arabinose, trehalose, salicin, xylose, mannitol, and sorbitol. Each isolate was also inoculated into a medium containing ornithine to detect the activity of the enzyme ornithine decarboxylase.

II. Growth Studies

Cultures

Source and origin of the cultures used in the studies are provided in Table 6.

Table 6. Details of the cultures used in the growth experiment

Culture	Origin	Serotype	Source
P. multocida (656)	Bovine	B:2	G. R. Carter, MSU[a], East Lansing, MI
P. haemolytica (P1148)	Bovine	1	G. R. Carter, MSU, East Lansing, MI
P. haemolytica (P3868)	Chicken	UT[b]	National Animal Disease Center, Ames, IA
Actinobacillus lignieresii	Bovine	---	Department of Microbiology, MSU, East Lansing, MI

[a]MSU = Michigan State University

[b]UT = untypable by IHA

Growth Experiment

The experiment was performed according to the method described by Wessman (1965) with minor modifications. The seed cultures were grown in brain heart infusion broth (Difco) for 16 hours and 0.5 ml of each of these cultures was transferred into 250 ml Erlenmeyer flasks containing 25 ml of brain heart infusion broth. Flasks were incubated at 37 C in a shaker water bath (Gyrotory Waterbath Shaker, Model G76). The number of viable cells was estimated by spreading 0.1 ml of diluted (10^{-7} to 10^{-9}) cultures into petri dishes containing tryptose agar (Difco). Following incubation at 37 C for 18 hours, the colonies were counted in a colony counter. Viable counts were made at 2-hour intervals up to 16 hours of incubation, at which time the experiment was concluded.

Turbidimetric measurements of growth were made by reading optical densities on a Spectronic-20 colorimeter (Bausch and Lomb) at 575 nm wavelength. Readings were made on cultures diluted 1:5 in distilled water with diluted medium as the blank.

III. Pathogenicity Studies

Pathogenicity to Mice

Cultures. Two bovine cultures and 6 avian cultures were injected into mice along with a mucin or hemoglobin preparation. The preparation of the mucin and hemoglobin suspensions is given below. Details of the cultures that were used in the studies are listed in Table 7.

Preparation of mucin. Seven grams of swine gastric mucin (Sigma) powder were blended vigorously for 2 to 3 minutes with 93.0 ml of distilled water. Mucin powder was added slowly to the blender to

Table 7. Origin and source of the cultures of *P. haemolytica* used in the pathogenicity studies

Culture	Origin	Serotype	Source
J-28	Bovine lung	2	National Animal Disease Center, Ames,IA
P-1148	Bovine lung	1	G. R. Carter, MSU,[b] East Lansing, MI
P3868 and P3873 (2 cultures)	Chicken lung	UT[a]	National Animal Disease Center, Ames, IA
A113 and A355 (2 cultures)	Chicken lung	UT	VDL,[c] MSU, East Lansing, MI
A289 and A283 (2 cultures)	Chicken	UT	VDL, MSU, East Lansing, MI

[a]UT = untypable by IHA

[b]MSU = Michigan State University

[c]VDL = Veterinary Diagnostic Laboratory

facilitate uniform mixing. The mucin mixture was placed in a heated "Magnastir" and stirred continuously until the temperature reached ca. 100 C. Finally, the mixture was autoclaved for 20 minutes at 121 C, after which the pH was adjusted to 7.2 $\pm$ 0.2 with the addition of 1 N sodium hydroxide.

Preparation of hemoglobin. One hundred milliliters of bovine blood were collected in a clean bottle and allowed to clot. After pouring the serum out, the clot was washed twice with 100 ml of normal saline. One hundred milliliters of distilled water were then added to the clot and the clot was broken up with a glass rod. After 5 minutes, the fluid portion was poured from the bottle and filtered through

0.45 μ (Nalge) membrane filters. The preparation was stored in the refrigerator until used. The concentration of hemoglobin in the preparation was measured in a hemoglobinometer (Coulter Electronics, Inc.).

Inoculation of mice. Cultures were grown in tryptose broth (Difco) for 16 hours at 37 C and 10-fold serial dilutions of the cultures were made in sterile normal saline. Dilutions ranging from 10^{-7} through 10^{-9} were used only for viable cell counts, whereas the rest of the dilutions (10^{-1} through 10^{-6}) were used for the determination of LD_{50} in mice. Viable cells were estimated by spreading 0.1 ml of diluted cultures onto the tryptose agar plates as described previously.

Adult, male, Swiss Webster albino mice, 6 in each group, were selected and caged separately. Undiluted and diluted cultures (0.25 ml of each) were injected intraperitoneally to each group of mice along with 0.25 ml of mucin or hemoglobin preparation. The culture and hemoglobin preparation were drawn separately into a syringe, mixed and inoculated immediately to avoid the deleterious effect of the hemoglobin preparation on the bacteria. Two more groups of 6 mice were injected intraperitoneally with mucin and hemoglobin preparations in 0.25 ml quantities to serve as toxicity controls. Culture control mice received 0.25 ml of each of the undiluted cultures intraperitoneally. Mice were observed daily for 14 days following inoculation. The intraperitoneal LD_{50} for each strain of *P. haemolytica* was calculated by the method of Reed and Muench (1938).

Pathogenicity to Chickens of Different Age Groups

Cultures. Three isolates of *P. haemolytica* of avian origin were used in this study. Isolates P3868 and A113 were isolated from chicken lungs, whereas the isolate A283 was isolated from chicken intestine.

Inoculation of chickens. Twelve-hour-old tryptose broth cultures with a viable cell count of 10^9 cells per milliliter were inoculated into White Leghorn males of 3 age groups. The route of inoculation, dose of inoculum, and the number of chickens per group are listed in Table 8. Chickens were observed daily for 3 weeks, at which stage the experiment was concluded. Dead birds were necropsied and lung, liver, heart blood and intestines were collected for microbiological evaluation. Those chickens that survived for 3 weeks were sacrificed by cervical dislocation and necropsied. Lung and intestine were collected aseptically for microbiological evaluation.

IV. Electron Microscopic Studies

Cultures

The cultures that were used for growth study experiments were used for electron microscopic studies. Details of the cultures are listed in Table 6.

Preparation of Cultures for Ultrathin Sections

Cultures were grown on tryptose agar plates for 12 hours at 37 C. Ten to fifteen milligrams of wet bacterial mass were washed twice in normal saline and finally suspended in 5 ml of glutaraldehyde phosphate buffer (1 ml of 25% glutaraldehyde + 9 ml of phosphate buffer) of pH 7.2. Following overnight incubation at 25 C, the buffer was removed by

Table 8. Inoculation details in chickens for the pathogenicity test of *P. haemolytica*

Isolate	Route of inoculation	Dose of Inoculum in ml			No. of chickens[a]	Control[a]
		Day-old	3 wk.	6 wk.		
P3868	intramuscular	0.25	0.5	0.75	30	30
	wing web	0.1	0.2	0.3	30	
	cloacal	0.25	0.5	0.75	30	
	oral	0.25	0.5	0.75	30	
A283	intramuscular	0.25	0.5	0.75	30	30
	wing web	0.1	0.2	0.3	30	
	cloacal	0.25	0.5	0.75	30	
	oral	0.25	0.5	0.75	30	
A113	intramuscular	0.25	0.5	0.75	30	30
	wing web	0.1	0.2	0.3	30	
	cloacal	0.25	0.5	0.75	30	
	oral	0.25	0.5	0.75	30	

[a]Ten chickens in each age group

centrifugation at 10,000 x g for 5 minutes. The culture pellet was suspended in 2 ml of 1% molten Noble agar (Difco) and poured onto a 3 x 1 inch glass slide. Agar was cut in small pieces and suspended in Zetterqvist's osmium. Details of fixation and epoxy embedding procedures are outlined in Table 9.

Ultrathin sections were cut with a diamond knife on an LKB Ultratome and double stained with saturated uranyl acetate (Watson, 1958) and lead citrate (Reynolds, 1963). The grids were examined in a Zeiss-9 electron microscope at 60 KV.

Assay of Poly-β-hydroxybutyric Acid (PHB)

The assay of PHB was performed according to the method of Law and Slepecky (1961) using *Escherichia coli* and *Azatobacter vinelandii* as negative and positive controls, respectively. The details of the test cultures are listed in Table 6. The cells were grown in 10 ml of tryptose broth for 10 hours and were centrifuged in polypropylene centrifuge tubes (Ivan Sorvall, Inc.) which had been previously washed thoroughly with ethanol and hot chloroform to remove plasticizers. The cell paste was resuspended in a volume of commercial sodium hypochlorite solution (Clorox) equal to the original volume of medium. After 1 hour at 37 C, the lipid granules were centrifuged, washed with water, and then washed with acetone and alcohol. Finally, the polymer was dissolved by extraction with 3 small portions of boiling chloroform, the chloroform solution was filtered, and the filtrate was used for poly-β-hydroxybutyrate assay. For the standard, samples containing 5 to 50 µg of polymer in chloroform were transferred to clean test tubes. Ten milliliters of concentrate sulfuric acid was added to all the tubes containing known and unknown quantities of polymer. The tubes were

Table 9. Schedule for fixation and epoxy embedding for electron microscopy

Preparation		Time Period
Zetterqvist's osmium		2 hr
Zetterqvist's washing solution		10 min
Alcohol	50%	2 5 min changes
	75%	2 5 min changes
	95%	2 5 min changes
	100%	15 min
	100%	30 min
Alcohol:propylene oxide (2:1)		15 min
Alcohol:propylene oxide (1:1)		15 min
Alcohol:propylene oxide (1:2)		15 min
Propylene oxide	100%	15 min
	100%	30 min
Propylene oxide:EAM[a] (2:1)		1 hr
Propylene oxide:EAM (1:1)		1 hr
Propylene oxide:EAM (1:2)		1 hr
EAM		overnight
Tissues with EAM in blocks		1-24 hr[b]
Blocks in oven at 60 C		48 hr[c]

[a]Epon-araldite mixture (EAM): Araldite 6005 - 20 parts Epon 812 - 25 parts dodecenyl succinic anhydride - 60 parts 2,4,6 tri(dimethylaminomethyl)phenol - 2 parts.

[b]Until the tissue has sunk to the bottom of the EAM

[c]Until EAM was completely polymerized

capped with glass marbles and heated for 10 minutes at 100 C in a water bath. The solution was cooled and, after thorough mixing, a sample was transferred to a silica cuvette and the absorbance at 235 nm was measured in a spectrophotometer (Varian, Cary 219) against sulfuric acid blank.

V. Serological Studies

Cultures

These included 59 avian isolates, as described previously in Table 5, and 50 mammalian isolates of *P. haemolytica*. Lyophilized strains of 12 *P. haemolytica* serotypes were obtained from G. H. Frank, National Animal Disease Center, Ames, Iowa. Source and origin of mammalian isolates of *P. haemolytica* are listed in Table 10.

Table 10. Source and origin of mammalian isolates of *P. haemolytica* used for serological typing

Number of isolates	Origin	Source
16	Bovine	J. R. Beck, American Cyanamid Company
3	Ovine	J. R. Beck, American Cyanamid Company
7	Bovine	G. R. Carter, Michigan State University
2	Bovine	G. H. Frank, National Animal Disease Center
9	Ovine	G. H. Frank, National Animal Disease Center
9	Ovine	E. L. Biberstein, University of California-Davis
1	Caprine	E. L. Biberstein, University of California-Davis
3	Bovine	E. L. Biberstein, University of California-Davis

Typing Antisera

Antiserum to each of the 12 serotypes and to each of the 6 avian isolates was prepared in the rabbits as described by Biberstein et al. (1960), except that the tryptose agar grown cultures were used. Smooth colonies of *P. haemolytica* were streaked onto the tryptose agar plates. Following 18 hours of incubation at 37 C, the growth was harvested with 10 ml of normal saline. The cells were killed with 0.3% formalin and stored in the refrigerator until used.

Two adult healthy New Zealand White rabbits were used for each antigenic preparation. The immunizing schedule used was that recommended for *Klebsiella pneumoniae* (Edwards and Ewing, 1955): 0.5 ml subcutaneously, then 1.0, 2.0, 3.0, 3.0, 3.0 ml intravenously at 4-day intervals. Six days after the final 3 ml dose, the rabbits were trial-bled. If the titers were satisfactory (>1:200), the serum was harvested on the following day. If not, 3 additional injections of 3.0 ml were given until a satisfactory titer was obtained. Antisera were stored at -60 C.

Indirect Hemagglutination (IHA) Procedure

The IHA procedure adapted to a microtiter system was used as described by Biberstein (1978). The isolate to be typed was grown in 5 ml brain heart infusion broth at 37 C for 16 hours. The culture was heated at 56 C for 30 minutes to kill the bacteria and to release soluble antigen from the surface of the cells into the medium. Bovine red blood cells (BRBC) were washed 3 times in phosphate buffered neutral formalinized normal saline solution and packed after the third washing. Packed BRBC (0.5 ml) was suspended in 5 ml of heated culture preparation. After thorough mixing, the culture-BRBC mixture was incubated in a 37 C water bath for 1 hour. At the end of the incubation period, the cells were

washed again 3 times in buffered formal saline. After the last washing, 10 ml of normal saline was added giving a 0.5% suspension of modified BRBC.

With a microtiter set, microtiter pipettes delivering drops of 0.05 ml were used. One drop of diluted (1:50) serum and 1 drop of modified BRBC suspension were placed in each well and the mixture was incubated at room temperature for 3 hours before being examined for hemagglutination. A positive test was indicated by a smooth, uniform layer of RBC's evenly lining the bottom of the wells. Dense red buttons at the lowest point of the wells indicated a negative test. With sera of acceptable titer (>1:200), these reactions were usually quite unequivocal and only 1 serum reacted with the culture to be identified. An antigen control well contained RBC suspension and normal saline.

Rapid Plate Agglutination (RPA) Procedure

The RPA test was performed by the method described by Frank and Wessman (1978). A drop of antiserum (approximately 10 µl) was placed on a clean glass surface, and then a small amount of *P. haemolytica* colony from blood agar was picked up on an inoculating needle and mixed with the serum. A strong positive reaction in the form of clumping and clearing occurred as the mixture was stirred with the needle. Negative reactions remained turbid. Tests were performed at room temperature All cross reactions were quantitated by titration of antisera against the cross reacting serotypes. In the control test, a drop of normal saline was mixed with a small amount of colony to be typed.

Titration of Antisera

Two-fold serial dilutions of the sera were made in normal saline containing 1:10,000 thimerosal. The IHA and RPA tests were performed in the same manner as described previously. Titers were expressed as the reciprocal of the last serum dilution at which positive agglutination occurred.

Counterimmunoelectrophoresis (CIE)

The CIE test was performed by the method of Cho and Greenfield (1978) as described by Carter and Chengappa (1981). Cultures were streaked to provide nearly confluent growth on fresh blood agar plates consisting of trypticase soy agar (BBL) with 6% ox blood. After incubation for 24 hours, the growth was washed off with 5 ml of normal saline. The suspension was heated at 56 C for 30 minutes, after which the bacteria were sedimented by centrifugation. The clear supernatant fluid was used as the capsular antigen.

The electrophoresis plates were prepared by precoating glass plates (10 x 8 cm) with 15 ml volumes consisting of 0.5% agarose (Seakem), 0.5% bacto agar (Difco) and 0.015% sodium azide in 0.025 M barbital buffer pH 8.8 (High Resolution Buffer; Gelman). Wells, 3 mm in diameter, were prepared with a template (Grafar). The distance between wells center to center was 7 mm. A 20 µl quantity of capsular antigen was placed in the cathodal well and an equal quantity of antiserum was placed in the anodal well. The electrophoresis tank (Gelman) contained barbital buffer pH 8.8. Controls included 0.85% sodium chloride solution against antisera and capsular antigens. The antigens and antisera were electrophoresed for 30 minutes at 150 V. The plates were then examined for precipitation lines and the presence of a distinct line was interpreted as positive. After electrophoresis, the plates

were held in a 2% saline bath overnight to wash away nonspecific precipitate, dried, and stained with 0.1% amido black.

Staining Procedure

The plates were dried overnight at 37 C with a wet filter paper covering the agar surface. The dried plates were immersed in 0.1% amido black (0.1 g of amido black powder + 50 ml of 1 M glacial acetic acid + 50 ml of 0.1 M sodium acetate) for 10 minutes and decolorized with 1 M glacial acetic acid. Decolorization step was repeated several times until a clear background was evident. Finally, the plates were rinsed in distilled water, air dried and photographed.

Agglutination Absorption Test (AAT)

The AAT was performed according to the method described by Frank (1980). Cells were harvested from 20-hour tryptose broth cultures, washed in 0.15 M sodium chloride, and suspended in 0.15 M sodium chloride with 1:10,000 thimerosal. Three milliliters of cell suspension equivalent to 10 times an optical density of 0.5 at 575 nm was centrifuged at 4,340 x g for 30 minutes. The cell pellet was suspended in 0.5 ml of a 1:16 dilution of the antiserum to be absorbed. After 2 hours of incubation at 37 C with frequent shaking, the mixture was refrigerated overnight, and the cells were centrifuged from the serum at 4,340 x g for 30 minutes.

VI. Estimation of Guanine-Cytosine (GC) Ratio

Cultures

Fourteen cultures of *Pasteurella* sp. and 2 cultures of *Actinobacillus* sp. were used. Details of the cultures are listed in Table 11.

The deoxyribonucleic acid (DNA) samples were isolated according to the method of Bohácek and Mráz (1973). The 24-hour cultures were washed

Table 11. Source and origin of the cultures used for the estimation of GC ratio

Culture Number	Origin	Source[a]
P. multocida		
656	Bison	G. R. Carter, MSU, East Lansing, MI
P1235E	Bovine	G. R. Carter, MSU, East Lansing, MI
P. haemolytica		
Type-1	Bovine	NADC, Ames, IA
Type-2	Bovine	NADC, Ames, IA
Actinobacillus lignieresii	Bovine	Dept. of Microbiology, MSU, East Lansing, MI
Actinobacillus equuli	Equine	Dept. of Microbiology, MSU, East Lansing, MI
P. haemolytica		
P3868	Avian	NADC, Ames, IA
P3873	Avian	NADC, Ames, IA
T3	Avian	Dept. of Animal Sciences, MSU, East Lansing, MI
A164	Avian	VDL, MSU, East Lansing, MI
A161	Avian	VDL, MSU, East Lansing, MI
A283	Avian	VDL, MSU, East Lansing, MI
A355	Avian	VDL, MSU, East Lansing, MI
A113	Avian	VDL, MSU, East Lansing, MI
A1681	Avian	VDL, MSU, East Lansing, MI
A289	Avian	VDL, MSU, East Lansing, MI
Escherichia coli[b]		
ATCC 14763	---	Dept. of Microbiology, MSU, East Lansing, MI

[a] MSU = Michigan State University, NADC = National Animal Disease Center, VDL = Veterinary Diagnostic Laboratory

[b] Control with a GC ratio of 50%

by SE solution (0.15 M sodium chloride + 0.1 M ethylenediaminetetraacetic acid, pH 8), centrifuged and washed twice with SE buffer again. For further treatment, the amount of 3 to 5 g wet bacterial mass was used and suspended with careful stirring into 50 ml of the mentioned buffer. Dodecyl sulfate was added to adjust 1% concentration and the mixture incubated for 15 minutes under periodic stirring in water bath at 60 C. After cooling the very viscous lysate was diluted with SE solution to make 80 ml; a 20 ml portion of 5 M sodium perchlorate was added to reach a final 1 M perchlorate concentration essential for separation of proteins from the DNA. At this stage, the samples were left overnight.

For deproteinization, chloroform-isoamylalcohol mixture (24:1) was used at a quantity of approximately 1/3 of the original volume of the suspension. Then the treatment continued under intensive shaking for 30 minutes, performed on a mechanical shaker, and 10 minutes centrifugation carried out at 5000 x g. Finally, a fibrous DNA was precipitated with 1.5 volumes of 96% redistilled ethyl alcohol.

The fibrous DNA was slightly pressured to be free from ethyl alcohol excess and immediately dissolved in a 20 ml portion of 10-fold-diluted SSC (0.15 M sodium chloride + 0.015 M sodium citrate, pH 7). After complete dissolving, the SSC concentration of a mixture was adjusted by addition of 0.1 volume of 10-fold concentrated SSC and by addition of 50 µg/ml ribonuclease (Sigma). After 30 minutes, an enzymatic cleavage was carried out at 37 C, prior to repeatedly performed deproteinization with chloroform-isoamyl alcohol, until the protein interlayer occurring after centrifugation disappeared. Deproteinized DNA was centrifuged at 75,000 x g for 1 hour and a clear upper layer was reprecipitated by the gradual addition of ethyl alcohol until 1.5 total volume was reached. Then the DNA sample was dissolved in 10-fold diluted

SSC solution. The approximate DNA concentration of 1 mg/ml, overlayered with a drop of chloroform, was kept in the refrigerator.

Two methods were used for the DNA base determination. A fundamental method used was that of thermal denaturation performed in PE buffer medium (0.01 M sodium phosphate buffer + 0.001 M ethylenediaminetetraacetic acid, pH 7) with a spectrophotometer (Beckman DU). The cell holder compartment was thermostated at both sides by 2 thermospacers for circulating hot water from a U-10 ultra thermostat. The temperature was measured with a rod thermometer directly in one of the cuvettes. For the GC ratio calculations, the equation GC = (Tm - 51): 0.45 was employed. The width of transitional interval (ΔT) was recorded and the value $2\sigma = (\Delta T - 3)\ 2.5$ was calculated. The value 2σ served then to express graphically the heterogeneity of the sample (DeLey and VanMuylem, 1963).

As a second control method for determining the percent GC content in isolated samples of DNA, the method of Fredericq et al. (1961) was used. This method is based on the fact that the extinction ratio of 260:280 nm (E_{260}:E_{280}) measured in a medium of 0.1 M acetic acid (pH 3) is dependent on the percent GC content in DNA. The samples of DNA were dissolved in a PE medium in a concentration of approximately 2 mg/ml. Before measurement, 0.1 M acetic acid was added to the final concentration of DNA, 20 to 30 µg/ml. The measurements of optical density at 260 and 280 nm, respectively, were made with a type Varian, Cary 219 spectrophotometer.

RESULTS

I. Fermentation Studies

The 59 isolates obtained from various sources (Table 5) were identified as P. haemolytica by cultural and morphological characteristics and standard biochemical tests. It was observed that all the isolates produced acid(s) from glucose, lactose, sucrose and mannitol. Eighty-seven percent of the isolates produced acid(s) from trehalose and xylose. However, arabinose and sorbitol were attacked by 67 and 78% of the isolates, respectively. The details of the results of the tests are given in Table 12. The growth of the isolates on sheep blood agar plates was much superior with 5% of the air replaced with carbon dioxide. Twenty-four-hour colonies on sheep blood agar plate ranged from 1 to 2 mm in diameter. They were smooth, opaque, and pasty in consistency, with a large zone of β-hemolysis (Appendix A1). Recovery of viable organisms from cultures stored at room temperature was not possible after 4 to 5 days. Viability of the cultures was not appreciably affected when they were stored in defibrinated sheep blood at -60 C.

II. Growth Studies

Growth curves of all the 4 cultures are depicted in Figures 1 and 2. Lag periods ranging from 1.5 to 3 hours were observed for these cultures under constant aeration. The growth was considerably faster

Table 12. Results of the biochemical tests on 59 avian isolates of *P. haemolytica*

Test	Percent Positives
Triple sugar iron agar[a]	100
Oxidase	100
Nitrate reduction	100
Indol	0
Growth on MacConkey agar	100
Urease	0
Ornithine decarboxylase	0
β-hemolysis	100
Acid from glucose	100
Acid from lactose	100
Acid from sucrose	100
Acid from arabinose[b]	67
Acid from trehalose[b]	87
Acid from salicin	0
Acid from xylose	87
Acid from mannitol	100
Acid from sorbitol	78

[a]Acid butt, acid slant, no hydrogen sulfide and no gas production.

[b]Results were recorded after 10 days of incubation.

Figure 1. Growth curves of *P. multocida* (656) and *A. lignieresii* in aerated cultures.

Figure 2. Growth curves of aerated cultures of *P. haemolytica* of bovine and avian origin.

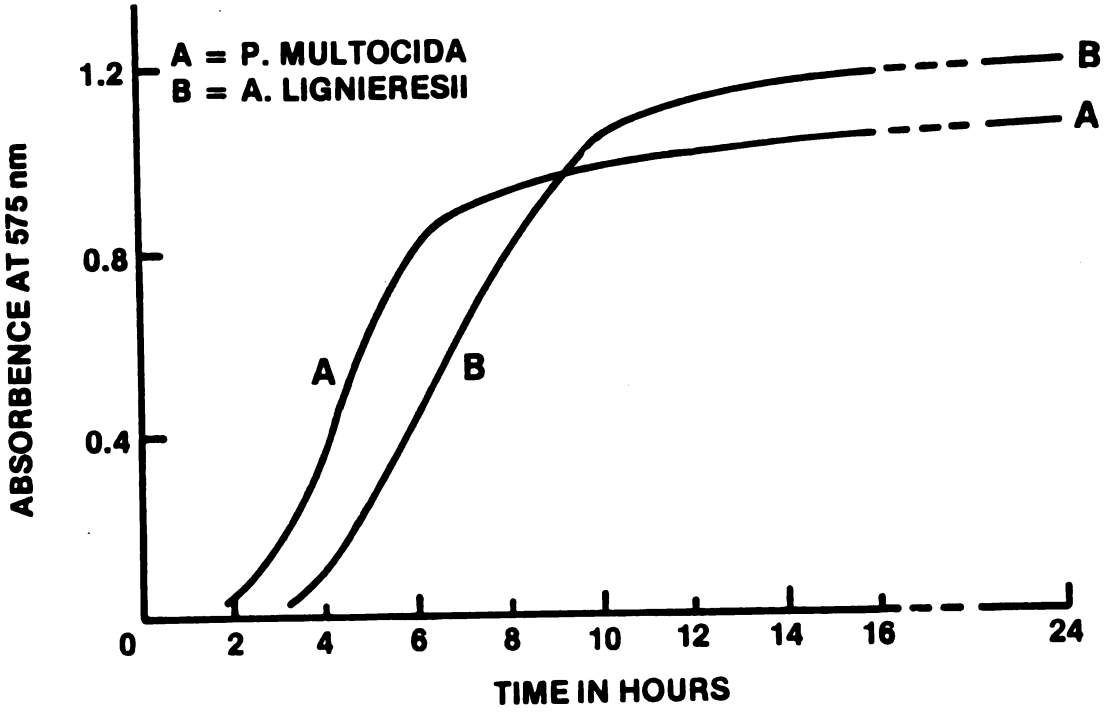

Figure 1

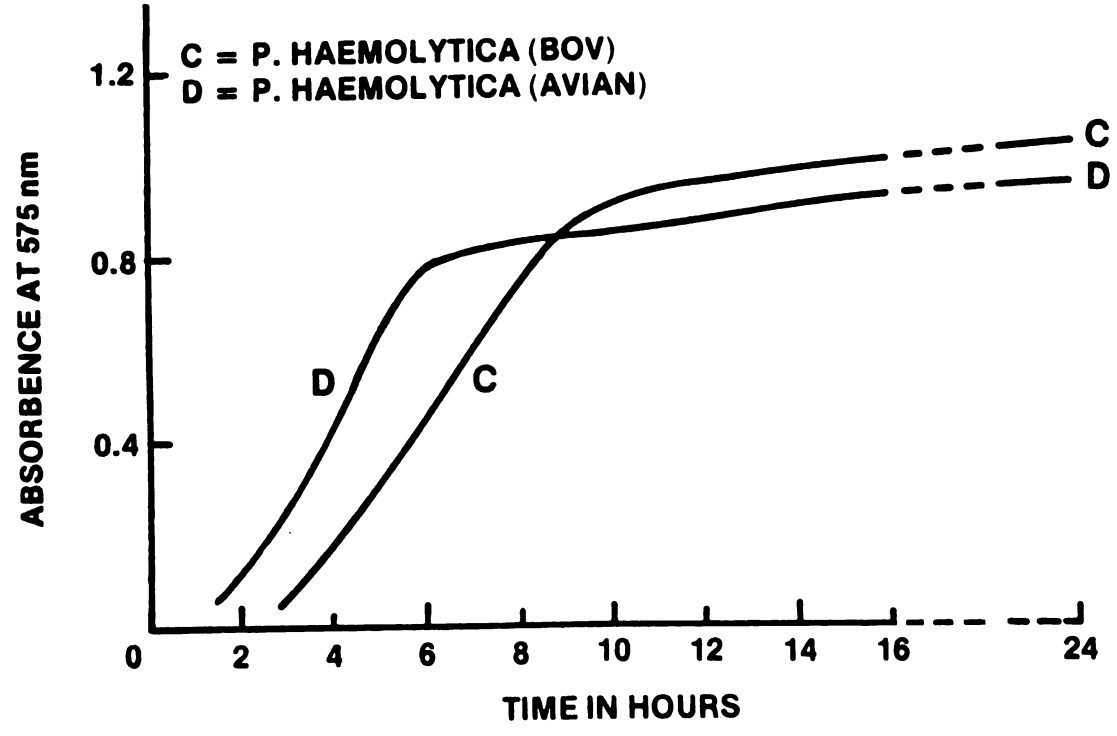

Figure 2

in *P. haemolytica* of avian origin than in the other 3 cultures studied. The avian isolate multiplied logarithmically for 1.5 to 6 hours, whereas the bovine isolate multiplied logarithmically from a period of 3 to 10 hours. The logarithmic phase of *P. multocida* was similar in length to that of *P. haemolytica* of avian origin. The longest logarithmic period was observed in *A. lignieresii*, viz. 12 hours.

The numbers of viable cells counted at different intervals are tabulated in Table 13. The figures are averages of counts of duplicate cultures. The maximum number of viable cells was observed at 7, 12, 10 and 6 hours of incubation of the cultures of *P. multocida*, *A. lignieresii*, *P. haemolytica* (bovine) and *P. haemolytica* (avian), respectively. The number of viable cells gradually declined in all cultures after the respective logarithmic peaks. The viable cell counts were not estimated after 16 hours of incubation.

III. Pathogenicity Studies

Pathogenicity to Mice

The preparation contained 2 grams of hemoglobin per 100 ml as measured in a hemoglobinometer. The results of the mouse pathogenicity study are summarized in Table 14. The hemoglobin preparation appeared to be a better enhancer of virulence of *P. haemolytica* than the mucin preparation, as there were more deaths of mice in hemoglobin-injected groups. However, isolate P3868 appeared to be more virulent when injected with mucin than with hemoglobin. A similar result was also observed with a hemoglobin solution of the same concentration prepared by lysing bovine red blood cells from defibrinated blood. No deaths were observed in toxicity and culture control groups. The viable cell count for each of the cultures was estimated by the spread plate

Table 13. Viable cell counts per milliliter of culture estimated at different intervals of incubation period

Period of Incubation	*P. multocida*	*A. lignieresii*	*P. haemolytica*[a]	*P. haemolytica*[b]
0	2.0×10^3	2.0×10^2	2.0×10^2	1.0×10^3
2	1.5×10^4	2.0×10^3	1.0×10^4	2.0×10^4
4	2.5×10^7	2.5×10^4	3.0×10^6	3.2×10^7
6	3.0×10^9	2.0×10^6	3.5×10^8	3.8×10^{10}
7	1.5×10^{10}	2.2×10^6	3.5×10^8	3.2×10^{10}
8	2.0×10^9	4.0×10^7	2.5×10^9	2.5×10^9
10	5.0×10^7	3.5×10^9	2.5×10^{10}	1.5×10^8
12	1.0×10^6	2.5×10^{10}	2.5×10^9	1.2×10^6
14	1.5×10^4	1.5×10^7	1.2×10^6	2.0×10^4
16	1.8×10^4	1.5×10^5	2.2×10^4	2.2×10^4

[a]Bovine isolate

[b]Avian isolate

Table 14. Mouse pathogenicity of *P. haemolytica* cultures injected with mucin and hemoglobin

Preparation	Viable Cell Count/ml[a]	LD_{50}	
		Dilution	No. organisms represented
J28 + mucin	2.5×10^9	$10^{-3.5}$	2.0×10^5
J28 + hemoglobin	2.5×10^9	$10^{-4.85}$	8.8×10^3
P1148 + mucin	2.8×10^9	$10^{-2.65}$	1.6×10^6
P1148 + hemoglobin	2.8×10^9	$10^{-3.65}$	1.6×10^5
P3873 + mucin	3.0×10^9	$10^{-1.0}$	7.5×10^7
P3873 + hemoglobin	3.0×10^9	$10^{-1.36}$	3.3×10^7
P3868 + mucin	3.5×10^9	$10^{-1.5}$	2.8×10^7
P3868 + hemoglobin	3.5×10^9	$10^{-1.0}$	8.8×10^7
A113 + mucin	3.2×10^9	$10^{-1.0}$	8.0×10^7
A113 + hemoglobin	3.2×10^9	$10^{-1.5}$	2.5×10^7
A355 + mucin	2.8×10^9	$10^{-1.2}$	4.4×10^7
A355 + hemoglobin	2.8×10^9	$10^{-2.0}$	7.0×10^6
A289 + mucin	3.0×10^9	$10^{-1.0}$	7.5×10^7
A289 + hemoglobin	3.0×10^9	$10^{-1.5}$	2.4×10^7
A283 + mucin	2.9×10^9	$10^{-1.0}$	7.3×10^7
A283 + hemoglobin	2.9×10^9	$10^{-2.25}$	4.1×10^6

[a]Viable cell count of the culture injected

technique as described previously, and the results are given in Table 14. The viable cell counts of the cultures of *P. haemolytica* ranged from 2.5×10^9 to 3.5×10^9 cells per ml. The total number of organisms represented by each LD_{50} was calculated and is presented in Figure 3. It was observed that mice injected with the culture-mucin mixture needed more viable cells to cause 50% mortality than with the culture-hemoglobin mixture. An exception was observed with culture P3868. Mice injected with this culture-mucin mixture needed fewer viable cells.

The two-way analysis of variance for the mouse pathogenicity experiment is presented in Table 15. The interaction and error were not separable. Accordingly, differences in treatment (mucin vs. hemoglobin) were not significant ($p>0.25$) and differences in strains (bovine vs. avian) were also not significant ($p>0.50$).

Table 15. Two-way analysis of variance for mouse pathogenicity test

Source of Variation	d.f.	m.s.
Treatments	1	2.39×10^{15}[a]
Strains	7	1.1107×10^{15}[a]
Error	7	3.6621×10^{15}

[a] $p>0.05$

Pathogenicity to Chickens of Different Age Groups

The intramuscular route of inoculation was the most effective route, followed by the wing web route, but the cloacal and oral routes were proved to be totally ineffective in causing deaths among day-old chicks.

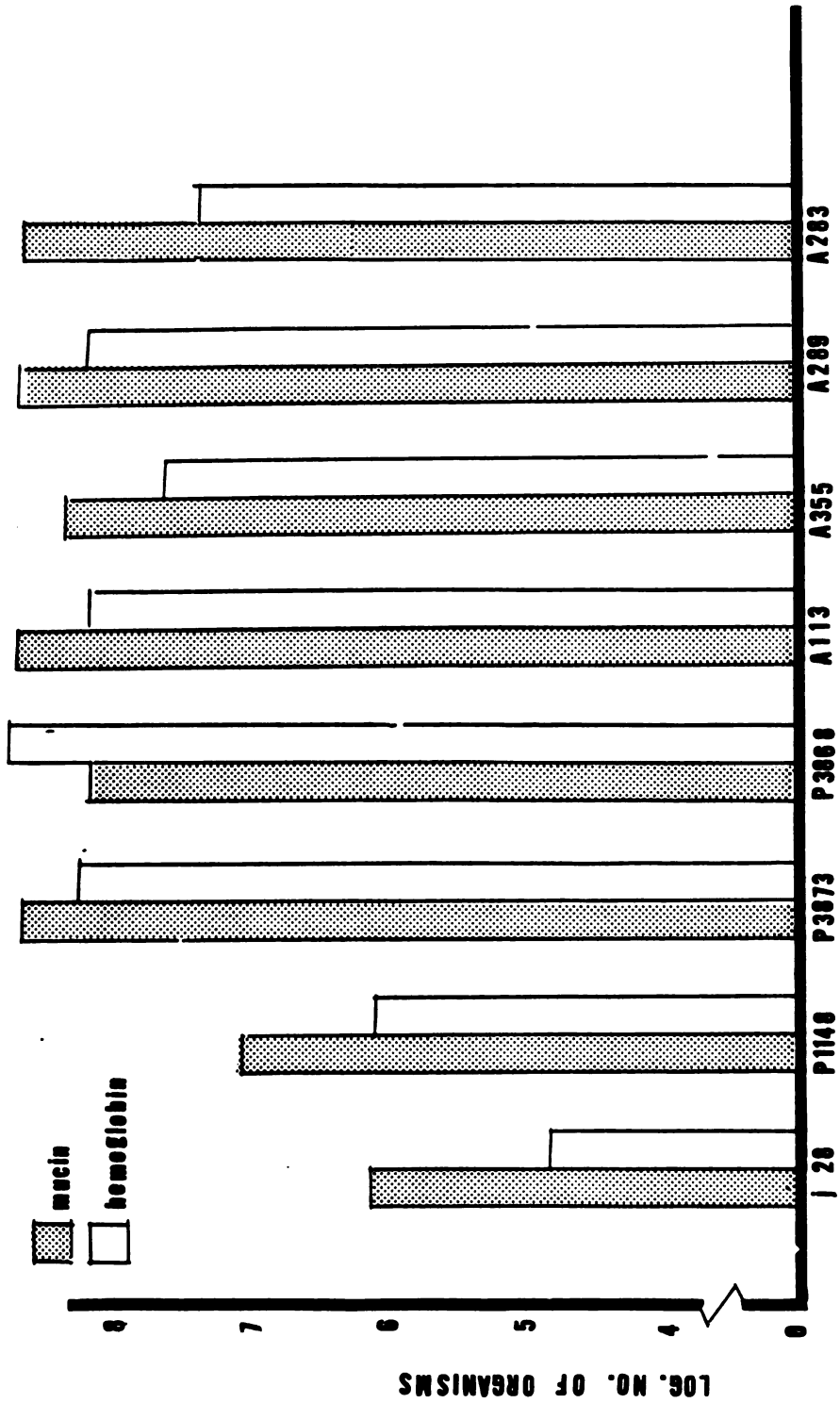

Figure 3. The number of viable organisms needed to cause 50% mortality among mice injected with mucin and hemoglobin preparations.

The deaths in day-old chicks for a 21-day postinoculation period are given in Table 16. There were 21 deaths observed in the day-old chick group inje-ted intramuscularly, but only 8 deaths were encountered in the same age group injected by the wing web route. Three- and six-week-old chickens demonstrated a strong resistance to the cultures, as there were no deaths or sickness observed for a period of 21 days.

The dead birds were necropsied and *P. haemolytica* was isolated from the lung and intestinal samples (Table 17). The remaining birds, including the controls, were sacrificed by cervical dislocation and necropsied. The lungs and intestines were cultured for *P. haemolytica* and the results are provided in Table 17. *Pasteurella haemolytica* was not isolated from the lungs and intestines of 91 uninfected birds and 8.3% of the 6-week-old birds carried *P. haemolytica* in their lungs. Although *P. haemolytica* was not isolated from the intestines of the 3-week-old birds, 8.3% of the intestines from the 6-week-old birds were positive for *P. haemolytica*. The day-old chicks that died of infection demonstrated a severe catarrhal enteritis and hemorrhages on the epicardia; livers and kidneys were swollen and dark red in color. The lungs were congested and tracheas were hemorrhagic in all the chicks that died of infection. The sacrificed birds, when necropsied, did not show any of the above-mentioned tissue changes.

IV. Electron Microscopic Studies

Electron microscopic examination of the cells grown on tryptose agar was carried out to measure the cell size and thickness of the cell membrane. The results are presented in Figures 4 through 7 and Appendices A-2 through A-5. The average cell size of each of the cultures was calculated and is listed in Table 18. It was observed that the length

Table 16. Results of inoculation of chicks with 3 cultures of *P. haemolytica* during a postinoculation period of 21 days

Isolate	Route of Inoculation	Mortality		
		Day-Old	3-Week	6-Week
P3868	Intramuscular	6/10	0/10	0/10
	Wing web	2/10	0/10	0/10
	Cloacal	0/10	0/10	0/10
	Oral	0/10	0/10	0/10
A283	Intramuscular	8/10	0/10	0/10
	Wing web	2/10	0/10	0/10
	Cloacal	0/10	0/10	0/10
	Oral	0/10	0/10	0/10
A113	Intramuscular	7/10	0/10	0/10
	Wing web	4/10	0/10	0/10
	Cloacal	0/10	0/10	0/10
	Oral	0/10	0/10	0/10

Table 17. Isolations of *P. haemolytica* from lung and intestinal samples of experimentally infected and control birds

Age Group	No. of Birds	Percent Recovery of *P. haemolytica*	
		Lung	Intestine
Infected birds (succumbed)			
Day-old	29	100	100
Infected birds (survived)			
Day-old	91	0	0
3-week	120	15	0
6-week	120	8.3[a]	8.3[a]
Controls			
Day-old	30	0	0
3-week	30	0	0
6-week	30	0	3.3

[a] *P. haemolytica* was not always isolated from the lung and intestine of the same bird.

Table 18. The average cell size and the cell membrane thickness of *Pasteurella* and *Actinobacillus* species as determined by electron microscopy

Organism	Size((μm)	Membrane$_o$Thickness (A)
P. multocida		
Type B (strain 656)	1.2±0.2X0.5±0.1	150
Type E (strain P1235E)	1.2±0.3X0.5±0.1	140
P. haemolytica		
Type-1 (strain P1148)	1.3±0.5X0.5±0.1	120
Type-2 (strain J28)	1.3±0.4X0.5±0.1	130
A. lignieresii	1.5±0.9X0.5±0.1	150
P. haemolytica[a]		
P3868	1.2±2.0X0.6±1.0	195
P3873	1.2±2.0X0.6±1.0	180

[a]Avian isolates

Figure 4. Electron micrograph of type B *P. multocida* (strain 656) grown on tryptose agar at 37 C. Arrows showing the bleb-like structures. Magnification X46,300.

Figure 5. Electron micrograph of type-1 *P. haemolytica* (strain P1148) grown on tryptose agar at 37 C. Arrow showing the bleb-like structure. Magnification X46,300.

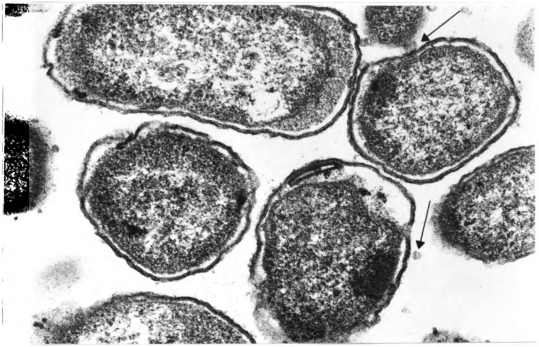

Figure 4

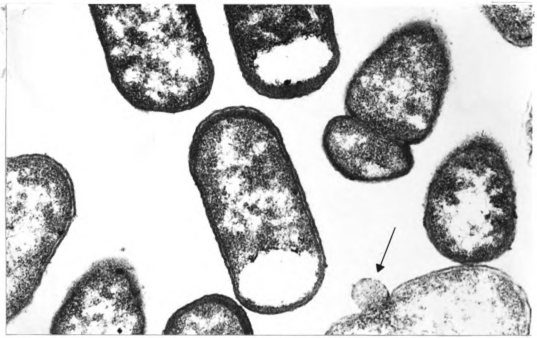

Figure 5

Figure 6. Electron micrograph of avian isolate of *P. haemolytica* (isolate P3868) grown on tryptose agar at 37 C. Arrow showing the bleb-like structures. Magnification X46,300.

Figure 7. Electron micrograph of *A. lignieresii* grown on tryptose agar at 37 C. Arrows showing the bleb-like structures. Magnification X46,300.

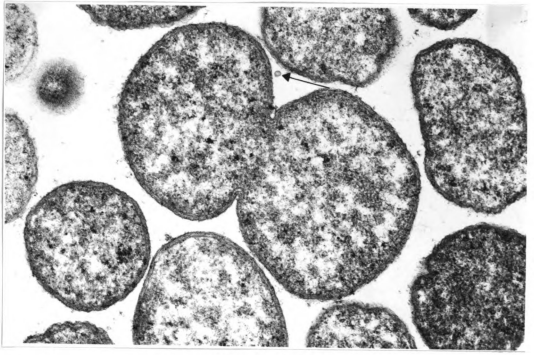

Figure 6

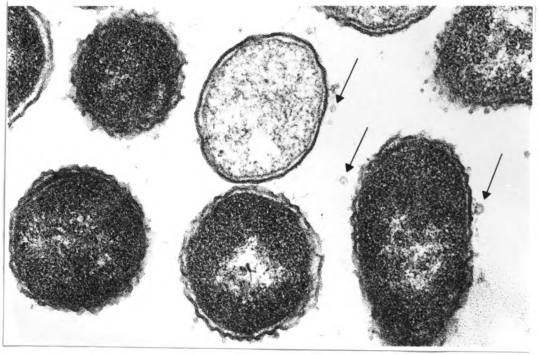

Figure 7

and width of avian isolates of *P. haemolytica* were slightly greater than those of bovine isolates. Similarly, the cell membrane thickness was found to be greater in avian isolates than in bovine isolates of *P. haemolytica*. The average cell membrane thickness was measured with the aid of an ocular micrometer and ranged from 120 to 195 Å. The cell membrane was tightly attached to the cell in avian isolates, but it was loosely attached to the cell in bovine isolates of *P. haemolytica* (Figures 5 and 6). The cell membranes of *P. multocida* and *A. lignieresii* were also found to be loosely attached (Figures 4 and 7). A few bleb-like structures were observed in all the micrographs. They were predominant with *P. multocida*, fewer with *A. lignieresii* and least with *P. haemolytica*. The bleb-like structures were fewer in avian isolates than in bovine isolates of *P. haemolytica*. There was no evidence for the presence of poly-β-hydroxybutyrate granules in the *Pasteurella* species studied (Table 19). A standard curve for poly-β-hydroxybutyric acid assay is provided in Appendix A-6.

V. Serological Studies

The antisera collected from the rabbits were titrated by IHA and RPA procedures and the titers are provided in Table 20. The RPA titers were lower than IHA titers for all the strains. The titers ranged from 1:256 to 1:1024 in the IHA test and 1:64 to 1:512 in the RPA test. Adverse effects during hyperimmunization of the rabbits were not observed.

IHA, RPA and CIE Tests

There were no cross reactions observed among 12 serotypes of *P. haemolytica* by the IHA test (Table 21). However, cross reactions were

Table 19. Spectrophotometric assay of poly-β-hydroxybutyric acid

Organism	Assay Result
P. multocida	
Type B (strain 656)	Negative
Type E (strain P1235E)	Negative
P. haemolytica	
Type-1 (strain P1148)	Negative
Type-2 (strain J28)	Negative
A. lignieresii	Negative
P. haemolytica[a]	
P3868	Negative
P3873	Negative
E. coli[b]	Negative
A. vinelandii[c]	Positive

[a]Avian isolates

[b]Negative control

[c]Positive control

Table 20. IHA and RPA titers of rabbit antisera against homologous *P. haemolytica*

Strain[a]	Capsular Type	IHA Titer	RPA Titer
J29	1	256	128[d]
J28	2	256	128
863	3	256	64
S	4	512	64[d]
G13	5	512	128
A30	6	256	64
H1	7	512	256
H21	8	512	256
B1	9	1024	512[d]
JF2	10	512	128
KC282	11	256	128
S209	12	512	256[d]
P3868	UT[b]	NA[c]	512[d]
P3873	UT	NA	512
A71	UT	NA	512[d]
A164	UT	NA	512
A161	UT	NA	512[d]
A283	UT	NA	512

[a]The last 6 strains are avian origin.

[b]UT = chicken isolates are untypable by IHA.

[c]NA = no agglutination.

[d]Flakes observed during agglutination.

Table 21. IHA, CIE and RPA reactions of rabbit antisera against heterologous and homologous *P. haemolytica* serotypes before and after absorption

Anti-serum	Antigen(s) Reacted With:[a] IHA	CIE	RPA	Antigen(s) Reacted With:[b] IHA	CIE	RPA
Type 1	1	1 & 4	1, 4 & 6	1	1	1, 6 & 8
Type 2	2	2	2 & 4	2	2	2 & 4
Type 3	3	3	3	3	3	3
Type 4	4	4 & 2	4, 2 & 8	4	4	4 & 8
Type 5	5	5	5 & 8	5	5	5 & 8
Type 6	6	6	6	6	6	6
Type 7	7	7	7 & 12	7	7	7 & 12
Type 8	8	8	8	8	8	8
Type 9	9	9, 2 & 6	9, 2 & 6	9	9	9 & 4
Type 10	10	10	10	10	10	10
Type 11	11	11	11	11	11	11
Type 12	12	12	12	12	12	12

[a]Before absorption

[b]After absorption

common in the RPA and CIE tests. It was possible to eliminate the cross reactions in the CIE test by serum absorption technique but not in the RPA test (Figure 10; Table 21). Figures 8 and 9 illustrate the homologous counterimmunoelectrophoretic patterns of 6 avian isolates and 12 mammalian type strains of *P. haemolytica*, respectively. The counterimmunoelectrophoretic patterns of cross-reacting serotypes 1, 4, and 9, before and after the absorption of serum, are shown in Figure 10.

It was not possible to eliminate the problem of flakes in the RPA test, even after the culture was passed once through a mouse to assure capsulation. Table 22 provides the distribution of 49 mammalian isolates among 12 serotypes of *P. haemolytica* by IHA, CIE and RPA procedures. Type-2 was found to be the predoimant serotype, followed by type-1. The remaining serotypes were less frequent. Of the 50 isolates, 49 were typable and 1 was untypable as determined by IHA and CIE procedures. In the RPA procedure, 40 isolates were typable and 10 were untypable. None of the 59 avian isolates reacted with the 12 type sera as tested by IHA, RPA and CIE procedures. The sera raised against 6 avian isolates were tested for cross reactions by CIE procedure and the results are presented in Table 23. No cross reactions were observed among the 6 avian isolates when the CIE test was run for 30 minutes. Strong precipitin lines were observed in all these tests, except with isolate A161. The band with the latter culture was much less dense.

Table 24 shows the distribution of 57 avian isolates of *P. haemolytica* among 6 serotypes as determined by IHA, RPA and CIE procedures. The avian type-2 was the predominant serotype reacting with 19 of 59 avian cultures used in the study. Of the 59 isolates, 57 were typable and 2 were untypable by CIE procedure, whereas by IHA procedure all the 59 isolates were untypable. However, the RPA procedure demonstrated

Figure 8. Counterimmunoelectrophoretic patterns of 6 avian type sera against homologous antigens (A) and saline controls (B). Ag = antigen; Ab = serum; C = saline.

Figure 9. Counterimmunoelectrophoretic patterns of 12 mammalian type sera against homologous antigens. Ag = antigen; Ab = serum.

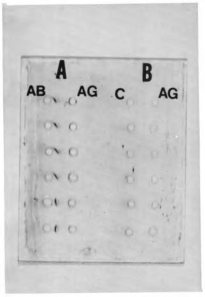

Figure 8

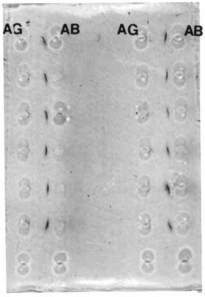

Figure 9

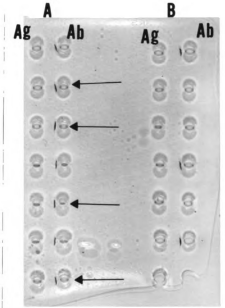

Figure 10. Counterimmunoelectrophoretic patterns of homologous and heterologous reactions of serotypes 9, 4 and 1, before (A) and after (B) absorption (Table 19). Ag = antigen; Ab = serum. Arrows show the heterologous reactions before the absorption of sera.

Table 22. The distribution of 50 mammalian isolates of *P. haemolytica* among 12 serotypes

Antiserum[a]	Test Procedure		
	IHA	CIE	RPA
Type-1	6	6	3
Type-2	21	21	16
Type-3	4	4	4
Type-4	1	1	1
Type-5	4	4	4
Type-6	1	1	1
Type-7	2	2	2
Type-8	3	3	3
Type-9	3	3	2
Type-10	1	1	1
Type-11	1	1	1
Type-12	2	2	2
Untypable	1	1	10

[a]Absorbed with the corresponding cross-reacting antigen(s).

Table 23. CIE reactions of rabbit antisera against heterologous and homologous *P. haemolytica*

Antiserum	Antigen					
	P3868	P3873	A164	A71	A283	A161
P3868	+	-	-	-	-	-
P3873	-	+	-	-	-	-
A164	-	-	+	-	-	-
A71	-	-	-	+	-	-
A283	-	-	-	-	+	-
A161	-	-	-	-	-	+[b]

[a] Avian isolates

[b] Weak line of precipitin

Table 24. The distribution of 59 avian isolates of *P. haemolytica* among tentatively designated 6 serotypes

Antiserum	Tentative Designation	Test Procedure		
		IHA	CIE	RPA
P3868	Type-1	0	13	56
P3873	Type-2	0	19	56
A164	Type-3	0	7	56
A71	Type-4	0	1	56
A283	Type-5	0	8	56
A161	Type-6	0	9	56
Untypable	---	59	2	3

that 56 isolates were typable and only 3 were untypable. None of the mammalian isolates reacted with the avian type sera either in CIE or RPA procedures. The RPA test failed to recognize any serotypes, even after diluting the sera (Table 24).

VI. Estimation of Guanine-Cytosine Ratio

For determining the percent GC content in the DNA samples of *Pasteurella* species and *Actinobacillus* species examined, 2 different methods were used. The values obtained by the 2 methods are provided in Table 25. The differences between the values obtained by the 2 methods were within the range of 0-4.2% GC (Table 25). The mean values of GC, obtained by thermal denaturation, were 39.65%, 40.85%, 41.0% and 40.25% for *P. multocida*, *P. haemolytica*((bovine), *P. haemolytica* (avian), and *Actinobacillus* species, respectively. Using the spectral analysis, the mean GC values were about 1% higher than the values obtained by thermal denaturation. *Escherichia coli* strain ATCC14763 used as control showed 49.5% GC content by thermal denaturation method and 49% GC content by spectral analysis method. The values 2σ and 3σ were calculated for each of the DNA samples and expressed graphically (Figure 11). The solid lines and the dotted lines correspond to 2σ and 3σ values, respectively.

Table 25. Guanine-cytosine content of *Pasteurella* and *Actinobacillus* species

Organism	Thermal Denaturation				Analysis of UV-Spectra	
	Tm (°C)[a]	GC%	ΔT[b] C	2σ[c]	$E_{260}:E_{280}$	GC%
P. multocida						
656	69.0	40.0	4.2	3.0	1.48	40.0
P1235E	68.7	39.3	4.6	4.0	1.46	41.2
P. haemolytica						
Type-1	69.2	40.5	4.4	3.5	1.48	40.0
Type-2	69.5	41.2	4.8	4.5	1.42	44.0
A. lignieresii	69.4	41.0	5.0	5.0	1.46	41.2
A. equuli	68.8	39.5	4.0	2.5	1.48	40.5
P. haemolytica[d]						
P3868	68.6	39.0	4.0	2.5	1.49	39.4
P3873	69.0	40.0	4.2	3.0	1.48	40.0
T3	70.2	42.4	4.0	2.5	1.44	42.5
A164	68.8	39.8	4.8	4.5	1.42	44.0
A161	69.8	41.8	3.6	1.25	1.45	41.8
A283	69.6	41.5	3.5	1.25	1.42	44.0
A355	70.0	42.0	3.8	2.0	1.45	41.8
A113	70.1	42.2	4.2	3.0	1.42	44.0
A289	69.2	40.3	3.8	2.0	1.44	42.5
E. coli ATCC14763	73.2	49.5	4.0	2.5	1.38	49.0

[a]Temperature of melting point

[b]Transition interval

[c]Includes about 95% DNA molecule

[d]Avian isolates

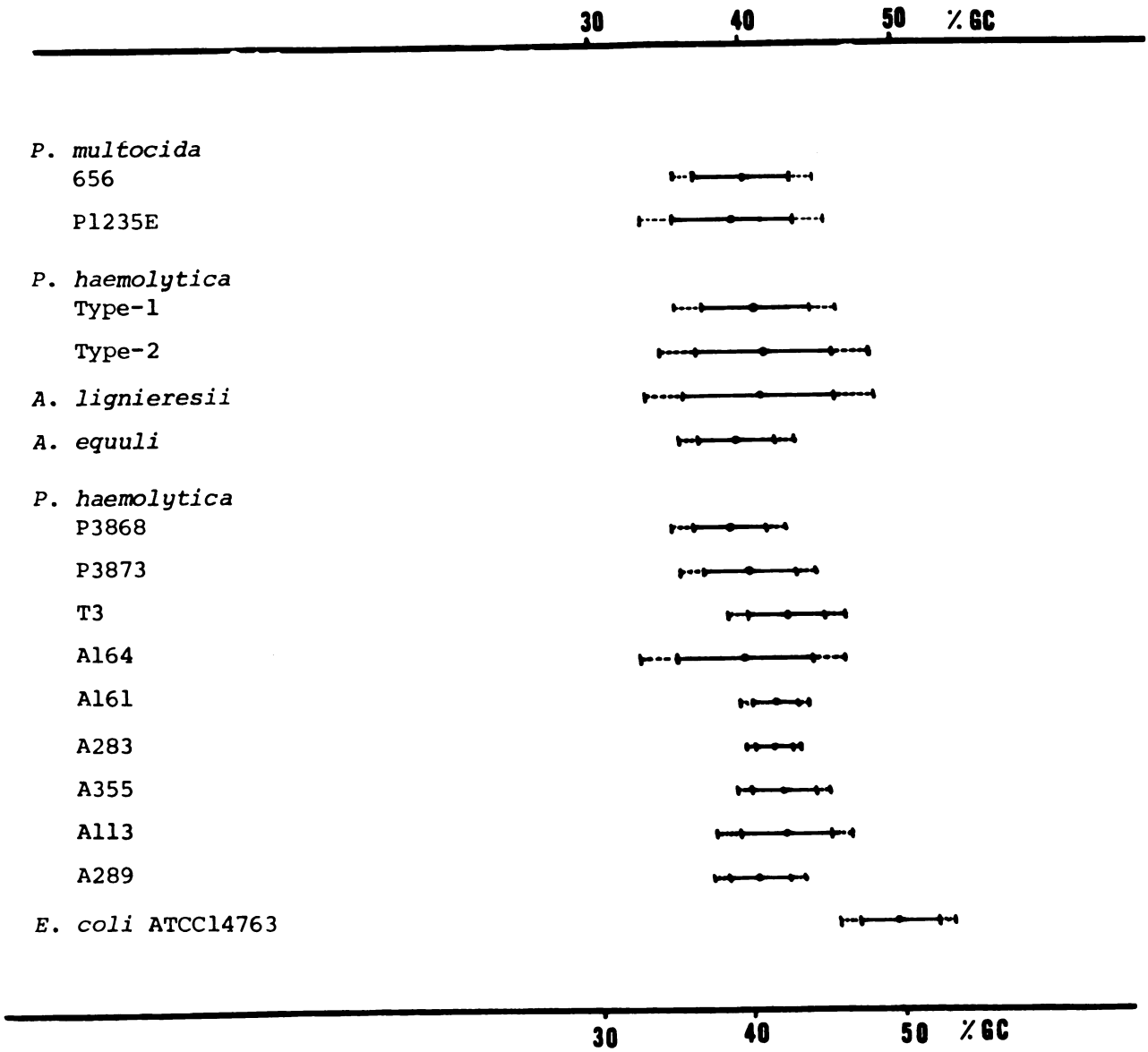

Figure 11. Graphic depiction of results of guanine-cytosine content of the DNAs of *Pasteurella* species, *Actinobacillus* species, and *E. coli*. The solid horizontal lines represent percent GC $\pm$ 2σ and the solid lines plus dotted lines represent percent GC $\pm$ 3σ.

DISCUSSION

I. Fermentation Studies

Considerable variations have been recognized in the fermentative activity of strains of *P. haemolytica*. Smith (1961) observed that all 14 of his type A strains of ovine origin fermented arabinose in 7 days, while 14 type T strains of ovine origin did not. He also found that the type T cultures fermented trehalose in 10 days, while the type A strains did not. However, Shreeve et al. (1970) reported that all T strains fermented trehalose but not arabinose, whereas only a portion of A strains fermented arabinose. The use of arabinose and trehalose to distinguish Smith's biotypes seems to be more reliable for T strains than for the A strains.

Fifty-two percent of the avian isolates used in the present study fermented both rehalose and arabinose in 10 days. This observation indicates that the avian isolates of *P. haemolytica* may fall into a separate biotype. This also suggests that the avian strains of *P. haemolytica* do not fit the original biotyping concept introduced by Smith (1961). Similar fermentative variations in avian isolates of *P. haemolytica* have been reported by Heddleston (1975), Harbourne (1962) and Janetschke and Risk (1970). Heddleston (1975) reported that all of his 20 avian isolates fermented trehalose in 10 days but not arabinose. However, in the present study, 87% of the cultures fermented trehalose and 67% of the cultures fermented arabinose in

10 days. This variation in fermentation reactions may have been due to the small number of avian isolates examined by Heddleston. Arabinose positive avian strains have also been reported by Janetschke and Risk (1970).

Although lactose negative A and T strains of *P. haemolytica* have been reported (Biberstein et al., 1960; Shreeve et al., 1970), none of the avian isolates was lactose negative. All the 59 isolates fermented lactose within 48 hours. Considerable fermentative variation from that of mammalian isolates was also observed among avian cultures in xylose, salicin and sorbitol by Shreeve et al. (1970), Biberstein and Gills (1962) and Smith (1961). Although variations were observed in fermentation reactions, the major genus characteristics were essentially identical.

The zone of β-hemolysis produced with avian isolates was much wider than that produced with bovine or ovine isolates. This finding is in agreement with Heddleston (1975) and Janetschke and Risk (1970). The zone diameter of hemolysis produced on bovine and ovine blood agar plates was not different with the avian cultures of *P. haemolytica*.

II. Growth Studies

The growth characteristics of bovine and ovine strains of *P. haemolytica* have been studied extensively by Wessman (1964, 1965, 1966) and Wessman and Wessman (1970), but no information is available on either the growth curves or nutritional requirements of avian isolates. Wessman (1966) reported that a strain of bovine *P. haemolytica* reached a count of 10^{10} cells per ml after 15 hours of incubation in a chemically defined medium. In the present study, the bovine isolate (P1148) of *P. haemolytica* reached a cell count of 2.5×10^{10} per ml after 10

hours of incubation in brain heart infusion broth. The difference was probably due to our richer medium. Although the culture was also different, there is not ordinarily much difference in growth capacity of different bovine strains. We observed that the avian culture P3868 had a maximum viable cell count, 3.8×10^{10} per ml, in a shorter period of incubation, viz., 6 hours, indicating that this strain probably has a shorter doubling time than mammalian strain.

There do not appear to be any reports in the literature on the growth curves of *P. haemolytica*; however, there have been several studies on the closely related species *P. multocida* (Banerji and Mukherjee, 1953; Handa, 1958; Carter and Bain, 1960). The growth curves show that the avian strain of *P. haemolytica* of this study had a shorter lag period (1.5 hours) than mammalian strains (3 hours). The avian strain multiplied logarithmically for 1.5 to 6 hours of incubation, whereas the mammalian strain multiplied logarithmically for 3 to 10 hours of incubation. This provides convincing evidence that the avian strain can grow faster than the mammalian strain of *P. haemolytica*. As with other bacteria, this would probably apply to the *in vivo* environment as well as the *in vitro* milieu. The well known hemorrhagic septicemia *P. multocida* culture 656 had a growth curve very similar to that of the avian *P. haemolytica*.

The suggestion has been made that *P. haemolytica* is more closely related to the *Actinobacillus* genus than to the *Pasteurella* and consequently should be excluded from the latter genus (Mráz, 1969). Our observations do not support the view that *P. haemolytica* should be transferred to the *Actinobacillus* genus.

III. Pathogenicity Studies

Pathogenicity to Mice

The low virulence of *P. haemolytica* for laboratory animals has hampered the acquisition of a suitable laboratory animal model to study infection by this organism. It has been necessary to include in the culture inoculum substances which effectively increase virulence. Gastric mucin was first used to enhance the virulence of *P. haemolytica* for mice by Smith (1958). The mode of action is still uncertain. Mucin has been shown to be anticomplementary *in vitro* by Lambert and Ritchley (1952), but a comparison of various mucins revealed little correlation between antiproperdin activity and the ability to promote infection with *E. coli* or *Staphylococcus aureus* (DeWitt, 1958). The introduction of high molecular weight dextran sulfates with the injected *P. haemolytica* increased the virulence to the same extent as gastric mucin, but a related compound (heparin) was less effective (Wessman, 1967). Although mucin has been used extensively in challenge experiments as an enhancer of virulence of *P. haemolytica*, the problem of nonspecific deaths due to mucin toxicity remains unsolved.

In the present study, a crude hemoglobin preparation was injected along with *P. haemolytica* as an enhancer of virulence. Toxicity to hemoglobin was not observed in the control group, and this lack of toxicity is considered to be a major advantage over the mucin preparation. Although the differences in virulence between mucin and hemoglobin injected groups were statistically not significant at the 5% confidence level, the LD_{50} showed a considerable difference between these 2 treatment groups. The results of the statistical analysis were not necessarily valid, as there were insufficient replications. We

observed that the hemoglobin preparation was a better enhancer of virulence than mucin with all the isolates studied except isolate P3868. In the latter, mucin was better. The exception may have been due to experimental error. Mammalian isolates were observed to be more virulent than the avian isolates of *P. haemolytica*, but the difference was not statistically significant at the 5% confidence level.

Iron compounds are known to increase the virulence of gram-positive and gram-negative bacteria including *P. multocida* (Bornside et al., 1968; Kochen et al., 1978; Bullen et al., 1966, 1967; Bjorn et al., 1979; Perry and Brubacker, 1979; Macham et al., 1975; Smith, 1977). Kaplan and Basford (1979) observed that the iron inhibited the leukocyte's phagocytosis-associated metabolic burst and specifically inactivated an important antibacterial product, hydrogen peroxide. They thought that the impairment of intraluekocytic bacterial killing in the presence of iron occurred by at least 2 mechanisms: (1) the inactivation of cationic proteins and (2) the destruction of hydrogen peroxide. Although we do not know the exact mechanism(s) by which *P. haemolytica* increases its virulence, it seems likely that one of the two mechanisms might have been operating in the presence of excess iron. *Pasteurella haemolytica* probably produced the iron-binding proteins which enabled the organism to acquire the circulating free iron. More work needs to be done to elucidate the role of free iron in the pathogenicity of *P. haemolytica*. Our work demonstrated that experimentally induced hyperferremia resulted in an increase in bacterial growth and virulence, with a marked reduction of the LD_{50}.

Pathogenicity to Chickens of Different Age Groups

Pasteurella haemolytica has been isolated from chickens and turkeys with respiratory diseases and salpingitis (Heddleston, 1975). The work of Janetschke and Risk (1970) indicated that the experimental infection of hens, chickens at 10 days of age, and adult white mice depended on the number of organisms administered. Our study revealed that the experimental infection with *P. haemolytica* depended on the route of inoculation and the age of the chickens. Day-old chicks were more susceptible than adult chickens. This was probably due to the poorer development of the immune system in the day-old chicks and thus a lesser ability to combat the infection. In the wing web method of inoculation, the dose of the inoculum was probably not enough to cause a high mortality among the day-old chicks. This was in agreement with the report of Janetschke and Risk (1970), who found that the infection was dose related. Although Harbourne (1962) reported experimental infection with *P. haemolytica* in 15-week-old Rhode Island Red chickens, we were unable to infect even 3-week-old White Leghorn chickens. These different results may have been due to the difference in the size of inoculum and route of inoculation used in these 2 studies. Also, the virulence of the organisms injected and the breed of chickens used for the studies might have added to this variation.

The incidence of isolation of *P. haemolytica* from the lungs and intestines of surviving birds was not high. This was further evident from the absence of gross pathologic lesions in surviving birds. It was not possible to confirm that those cultures isolated were the same as the original injected cultures. Our results indicated that the injected organisms were not eliminated from the body and that possibly there was a tendency for a carrier state being established in survivors. The

percent isolation of *P. haemolytica* from the control birds was considerably less as compared to the percent isolations from birds of the infected survival groups. This strongly suggests that a carrier state for *P. haemolytica* can be experimentally established in chickens.

IV. Electron Microscopic Studies

There do not appear to be any reports in the literature on the ultrastructure of *P. haemolytica*. The present study revealed few important structural differences between the avian and mammalian strains of *P. haemolytica*. The cell membrane of avian *P. haemolytica* was very tightly attached to the cell and was much thicker than that of mammalian *P. haemolytica*. The cell membranes of *P. multocida* and *A. lignieresii* were loosely attached like that of mammalian *P. haemolytica*. The cells of avian strains were found to be shorter and wider than those of mammalian strains of *P. haemolytica*. The results of this ultrastructural analysis suggested that the avian strains are slightly different from mammalian strains of *P. haemolytica*. The results also indicated that there were no major ultrastructural differences between *P. haemolytica* and *A. lignieresii*.

Projections of bleb-like material from the outer membrane were observed in the micrographs of all the cultures studied. The gram-negative bacteria that have been shown to release endotoxin in the form of cell wall blebs originating from the outer membrane include *E. coli* (Work et al., 1966), *Vibrio cholerae* (Chatterjee and Das, 1967) and *Neisseria meningitidis* (Devoe and Gilchrist, 1973). It has been shown that the pathogenicity of certain gram-negative bacteria depended on the release of endotoxin (Devoe and Gilchrist, 1973). Since *P. haemolytica, P. multocida* and *A. lignieresii* are known to produce these

bleb-like structures, it seems possible that the pathogenicity of these organisms may be related to the active extrusion of endotoxin as in other gram-negative bacteria.

Poly-β-hydroxybutyric acid has been recognized in many gram-positive and gram-negative bacteria (Duodoro-f and Stanier, 1959; Forsyth et al., 1958; Kallio and Harrington, 1960; Morris and Roberts, 1959). This polymer is thought to be the storage granule of some bacteria. *Pasteurella haemolytica, P. multocida* and *A. lignieresii* did not seem to produce this polymer. Its absence was confirmed by a spectrophotometric assay. The clear circular spaces observed in the cells of *P. haemolytica* of mammalian origin which were originally thought to be storage granules were probably artifacts produced as a result of the fixation.

V. Serological Studies

The titers presented in Table 20 suggest that the rapid plate agglutination test is less sensitive than indirect hemagglutination test. This is in agreement with Frank and Wessman (1978), the developers of the RPA test. Although Frank and Wessman (1978) did not apparently encounter the problem of "flakes" during the RPA test, we observed flakes in 7 of 18 homologous reactions. Since the elimination of the flakes was not possible, even after the cultures were passed once through mice, it was thought that the flake formations were due to partial autoagglutination. It was not possible to estimate the IHA titers of the sera of the avian strains, as there were no reactions observed. This is in agreement with Biberstein et al. (1960), who serologically examined 3 avian strains. The reasons for the negative IHA tests with the avian strains have not been elucidated and, as a

result, the taxonomic relationship of these strains to those of mammalian strains has been questioned (Biberstein, 1978).

IHA, RPA and CIE Tests

Biberstein et al. (1960) divided mammalian *P. haemolytica* into 10 serotypes with an IHA procedure. Later, this was expanded to 12 serotypes (Biberstein and Gills, 1962; Bibersten and Thompson, 1966). Recently, a rapid plate agglutination procedure for serotyping mammalian *P. haemolytica* was developed and the test yielded essentially the same results as the IHA procedure (Frank and Wessman, 1978). The CIE test has been used effectively to detect a variety of microbial antigens, including the capsular polysaccharides of *Haemophilus influenzae* (Ingram et al., 1979), pneumococci and meningococci (Shackelford et al., 1974) and *P. multocida* (Carter and Chengappa, 1981).

In the present study it was found that the CIE test is a rapid, specific method for serotyping mammalian *P. haemolytica*. The procedure worked well with older laboratory cultures as well as with those freshly recovered from clinical material. The CIE procedure yielded essentially the same result as the IHA procedure. The advantages of the CIE test over the IHA test were found to be as follows: (1) it was less time consuming; (2) there was no problem of hemolysis of red blood cells as experienced in the IHA test; and (3) it was a more sensitive test. The CIE test was also found to be better than the RPA test, as more mammalian isolates were typable. There were also fewer cross reactions and it was more sensitive than the RPA test. The cross reactions observed in the CIE test were eliminated after the sera were absorbed with the corresponding antigen(s), but it was not possible to eliminate the cross reactions in the RPA test. The cross reactions

were probably due to repeated injedtions of antigens during hyperimmunization procedures. We think that the problem of cross reaction in the CIE test might be eliminated by injecting rabbits with a single dose of antigen instead of 6 doses. Since the CIE test proved to be more sensitive, the antibody response produced by a single dose of antigen would probably be sufficient to yield a clear-cut specific line. The results of the RPA test suggested that the somatic antigens might have contributed to the reactions, as the size of the capsule of *Pasteurella* species is thought to be a phenotypic character and in some instances may have been deficient. The inconsistent reactions observed in the RPA test before and after the serum absorption are further supportive of the effect of somatic antigens on the test. The possible involvement of somatic antigens in the RPA test has been suggested by Frank and Wessman (1978).

Of the 50 mammalian isolates, only one was not typable by the IHA and CIE procedures, whereas in the RPA procedure 10 isolates were not typable. The distinct advantage of the CIE procedure, besides being rapid and specific, was that more untypable isolates would be typed.

The serotyping of avian strains of *P. haemolytica* by the IHA test was found to be unsuccessful (Biberstein et al., 1960). Frank and Wessman (1978) have not included the avian strains in their RPA procedure. The CIE procedure identified 6 different capsular types among the 59 cultures examined. In contrast, the 59 avian strains were not typable by the RPA or IHA procedures. Whether or not a modification of the IHA procedure will identify the 6 capsular types remains to be explored. Although agglutination was observed in the RPA procedure with avian strains, the test was not specific enough to identify the capsular

substances. The lack of specificity may have been due in part to the possible interference of somatic antigens.

VI. Estimation of Guanine-Cytosine Ratio

The GC ratios of *Pasteurella* and *Actinobacillus* species have been studied by Bohácek and Mráz (1967, 1973) and the results indicated that species of these 2 genera were closely related genetically. The genetic relatedness of *Pasteurella* species was also studied by DNA hybridization technique (Ritter and Gerloff, 1966), and the results indicated that the *Pasteurella* genus was made up of several subgroups whose members were closely related to each other. The results of our studies were in agreement with the results of previous workers. The present study indicated that the avian strains were genetically closely related to *P. multocida*, mammalian *P. haemolytica*, and *Actinobacillus* species. This further suggested that the avian isolates should be retained in the genus *Pasteurella*. The estimation of the GC ratio is thought to be a useful tool in taxonomic problems at the genus level but not at the species level.

The differences between the results obtained by the 2 methods indicated the presence of impurities in the DNA preparations. In our opinion, the method recommended by Frederieq et al. (1961) is useful for a rapid and orienting determination of percent GC. However, since this method does not furnish data on the heterogeneity of DNA molecules, it is advisable to use in parallel method of Tm determinations to obtain more detailed information.

Figure 11 illustrates graphically the base composition and exponential distribution of DNA molecules. The solid horizontal lines represent percent GC $\pm$ 2σ and the solid lines plus dotted lines

represent percent GC $\pm$ 3σ of the DNA molecules. When comparing the heterogeneity of molecular distribution of individual DNA samples, it is apparent that there is a remarkable genetic relationship among the strains studied.

CONCLUSIONS

The *P. haemolytica* cultures isolated from avian species were found to be different in their carbohydrate fermentive reactions from those of mammalian cultures. Fifty-two percent of the avian isolates fermented arabinose and trehalose within 10 days, indicating that they may represent a distinct biotype within the avian strains.

The growth studies revealed that the avian strain had shorter lag and logarithmic phases than that of the mammalian strain.

A crude 2% hemoglobin preparation injected into mice along with *P. haemolytica* was found to be a better enhancer of virulence than 7% swine gastric mucin. The lack of toxicity of the hemoglobin preparation was considered a major advantage over the mucin preparation in challenge experiments. The avian cultures appeared to be less pathogenic to mice than mammalian cultures. The difference in mouse virulence between the avian and mammalian isolates was not statistically significant, as there were insufficient replications. *Pasteurella haemolytica* was found to be pathogenic to day-old chicks and the pathogenicity depended on the dose and route of inoculation.

The ultrastructure of *P. haemolytica* indicated that the cell membrane of avian isolates was ca. 62 Å thicker than that of mammalian isolates. The study also indicated that the cell membrane of avian strains was loosely attached to the cell but in mammalian strains, in contrast, the cell membrane was tightly attached to the cell. The

bleb-like structures observed on the cells may have a direct relationship with the virulence of the organism, as these structures are known to extrude endotoxin.

The counterimmunoelectrophoresis (CIE) procedure was found to be less time consuming and more sensitive than the indirect hemagglutination (IHA) test. The CIE test yielded essentially the same result as the IHA test. The results indicated that the CIE test was a much better test than the rapid plate agglutination (RPA) test, as more mammalian isolates were typable and fewer cross reactions were observed. It was possible to serotype 57 avian isolates into 6 distinct capsular types by CIE. The IHA and RPA tests failed to identify these specific capsular substances.

The guanine-cytosine (GC) ratios of the DNA samples studied indicated that the avian strains were genetically closely related to *P. multocida*, *P. haemolytica* and *Actinobacillus* species. The pooled results of all the studies strongly support the contention that the avian isolates are different from the mammalian isolates of *P. haemolytica* and should perhaps be placed in a new group, viz., *Pasteurella avihaemolytica*.

Further research should be directed toward serological and fermentative studies with a larger number of avian strains isolated from different geographic regions. More work is also needed to elucidate the pathogenic mechanisms of avian strains under various stress situations. Studies on the effect of different iron compounds as enhancers of virulence of *P. haemolytica* could yield information which may also be helpful in understanding the *in vivo* pathogenic mechanisms. Research should also be directed to exploring the role of capsules and possibly the role of pili or other adherence structures in the attachment and colonization of *P. haemolytica* in the intestine and lungs. This

information may be useful in reducing the incidence and severity of *P. haemolytica* infection in domestic animals and poultry.

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APPENDIX

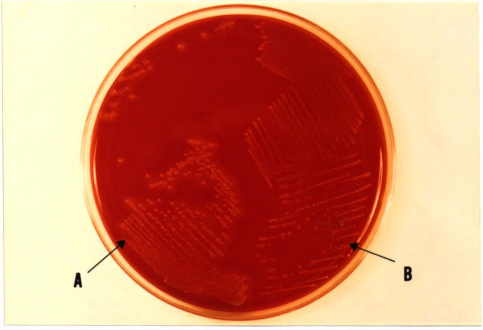

Figure A-1. Blood agar plate showing the β-hemolytic colonies of *P. haemolytica*. A = avian isolate; B = mammalian isolate.

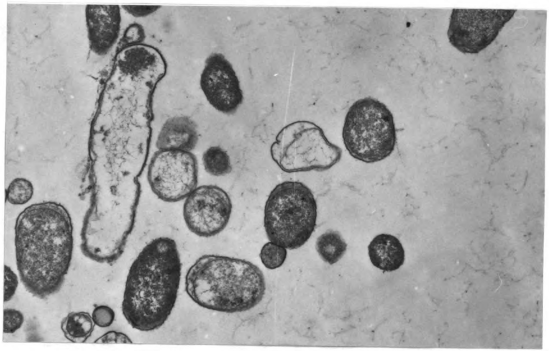

Figure A-2. Electron micrograph of type B *P. multocida* (strain 656) grown on tryptose agar at 37 C. Magnification X22,500.

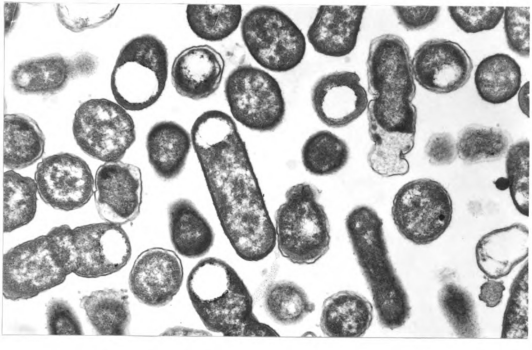

Figure A-3. Electron micrograph of type-1 *P. haemolytica* (strain P1148) grown on tryptose agar at 37 C. Magnification X22,500.

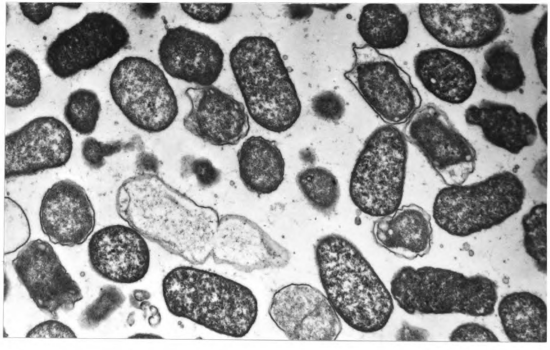

Figure A-4. Electron micrograph of avian isolate of *P. haemolytica* (isolate P3868) grown on tryptose agar at 37 C. Magnification X22,500.

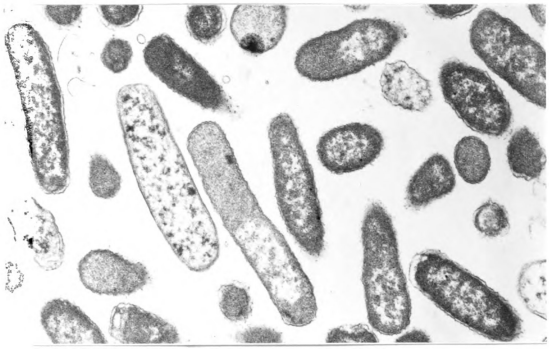

Figure A-5. Electron micrograph of *A. lignieresii* grown on tryptose agar at 37 C. Magnification X22,500.

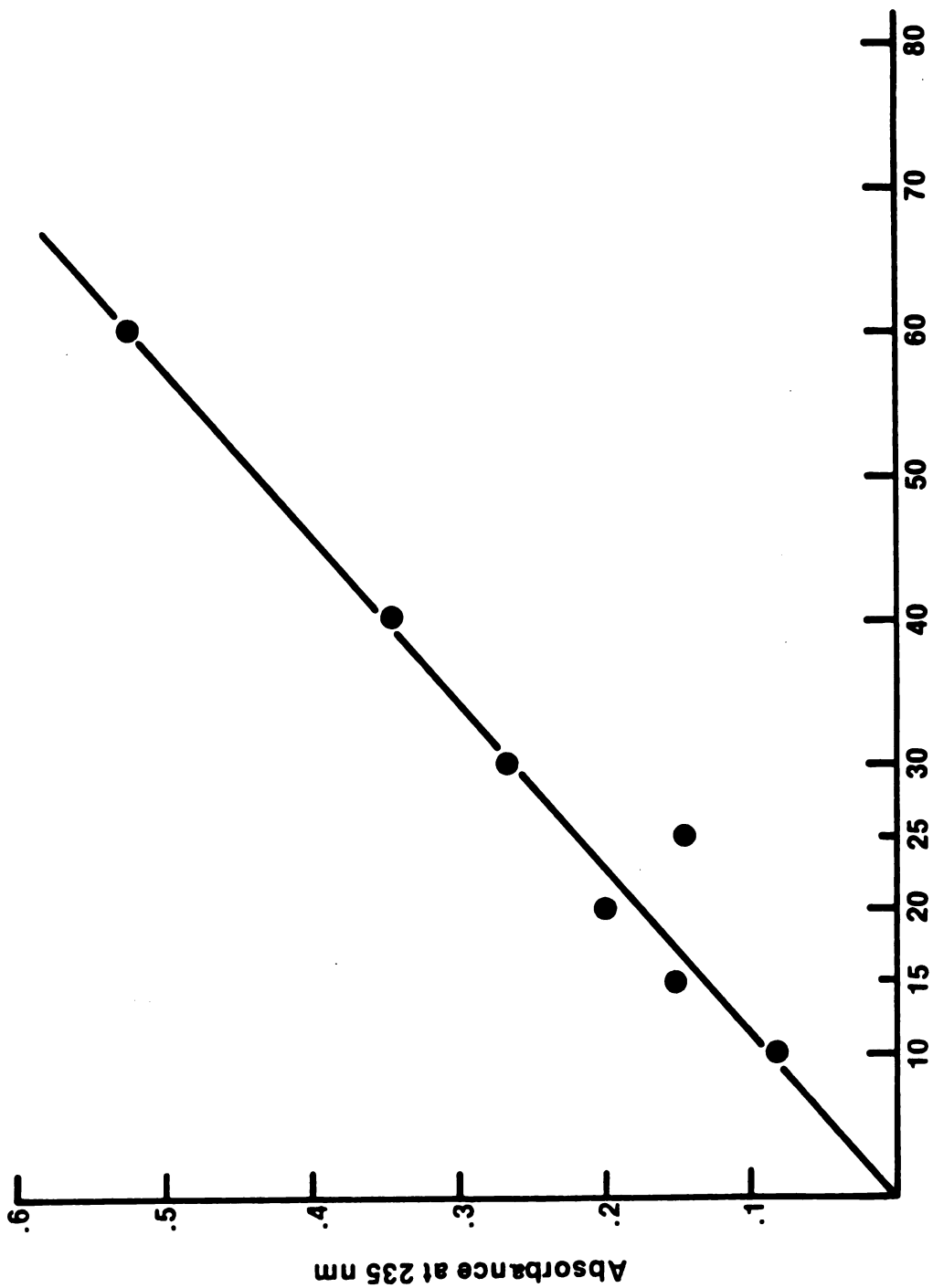

Figure A-6. A standard curve for poly-β-hydroxybutyric acid assayed by Law and Slepecky method.