STUDIES ON THE SEROLOGICAL RELATIONSHIPS, FERMENTATION REACTIONS, AND DISSOCIATION PATTERNS OF PASTEURELLA HEMOLYTICA ISOLATED FROM CHICKENS

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W. L. Stafseth
Major professor

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STUDIES ON THE SEROLOGICAL RELATIONSHIPS, FERMENTATION REACTIONS, AND DISSOCIATION PATTERNS OF PASTEURELLA HEMOLYTICA ISOLATED FROM CHICKENS

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MARY LOUISE MAYER

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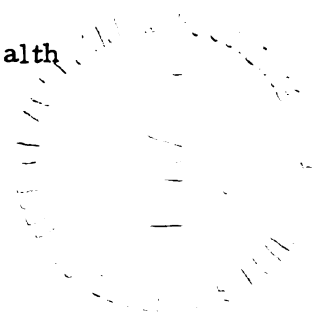

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TABLE OF CONTENTS

LIST OF TABLES

INTRODUCTION AND HISTORICAL REVIEW

While examining routine blood plates inoculated from diseased birds submitted to the poultry diagnostic laboratory at Michigan State College, conspicuous, hemolytic colonies were noted with significant frequency. These colonies were 1.5 to 3 mm in diameter, circular with an entire margin, convex, translucent, smooth and glistening, butyrous in consistency and cream colored. The average colony appeared much like a hemolytic streptococcus colony although many colonies were larger than a typical streptococcus colony. The cells were gram negative, short cocco-bacilli with marked tendency toward pleomorphism.

Attempts to identify the organism biochemically led to the conclusion that it was Pasteurella hemolytica, although no reference could be found describing its isolation from poultry or its significance in poultry.

The earliest reference in the literature to a hemolytic pasteurella was found in the work of Jones (1921). At that time it was customary to designate members of the hemorrhagic septicemia group of pasteurellae by host affinity. It was becoming evident, however, that host relationship was not the criterion for differentiation of

species; an organism isolated from one species was often pathogenic for another. Jones separated the organisms within the species then designated "bovisepticus" into three groups on the basis of biochemical and immunological differences. Prior to this time, fermentation tests to characterize the pasteurellae, or any organism, were not commonly employed. Immunological studies had been confined to attempts to protect laboratory animals with immune sera against small doses of virulent culture. For his studies Jones used strains of pasteurellae which he had isolated from (1) cows during an outbreak of pneumonia in a dairy herd in 1920, (2) sporadic cases in calves of the same herd following the main outbreak, and (3) strains, isolated by Theobald Smith in 1917-1920, associated with a previous outbreak of pneumonia in calves of the same herd. All the strains isolated by Jones during the enzootic of 1920, and from the sporadic cases following, were hemolytic and indol negative. They were isolated in pure culture. Primary infection in the animals examined by Smith was attributed to Bacillus actinoides. None of Smith's pasteurella cultures was hemolytic and all were indol positive.

Group I was composed of the eight strains isolated by Jones. These strains produced acid from dextrose, maltose, mannite, and sucrose giving a pH of 6.1 to 6.5 after incubation at 38 C for seven days, and acid from lactose giving a pH of 6.4 to 6.8 on primary

isolation. After several transfers on artificial media, lactose was fermented to the same extent as the other sugars. Indol was not produced. The organisms were insoluble in ox or guinea pig bile. Surface colonies were round, flattened, translucent, 3 to 5 mm in diameter in 48 hours, and hemolytic. All were encapsulated. Virulence was low for rabbits, mice, and cows; on subcutaneous injection only a slight local reaction resulted. When introduced into the lungs of cows, however, a severe diffuse pneumonia developed.

Group II was composed of the six strains isolated by Smith. They produced acid from dextrose and sucrose, but not from lactose, maltose, and mannite. The organisms produced indol. Broth cultures were soluble in ox or guinea pig bile within ten minutes to one hour. They were nonhemolytic, encapsulated, and relatively nonvirulent.

Group III comprised two strains: one strain was isolated by Smith at the beginning of the 1917 outbreak, and the other strain was isolated in another state in 1913. These were like Group II organisms except that they fermented mannite, were insoluble in bile, and were virulent for rabbits. Injection of 0.25 ml of a 24-hour broth culture killed rabbits in three to four days. Organisms were present in the blood 24 hours before death. The presence of capsules was variable in this group.

Immunological studies revealed groupings identical with the cultural groupings. Agglutinins for Groups I and II were produced with great difficulty to a titer of 1-200. Agglutinins for Group III were easily developed to a titer of 1-500. No cross-agglutination occurred between groups. Antigens were prepared by suspending growth from 24 or 48 hour slants in 0.85 per cent sodium chloride containing 0.25 per cent phenol. Tests were incubated at 38 C for 18 hours.

Jones concluded that Group I organisms differed enough to be considered a separate species, and proposed the name Pasteurella bovisepticus be used to identify them since infection occurred chiefly in adult cows. Group II and III organisms he considered varieties of the same species, and proposed they be called Pasteurella vituli-septicus. Although other serological types have since been identified within these groups, this classification of Jones still serves to separate the members of the hemorrhagic septicemia pasteurellae into their principal types. His work has been verified and extended to include organisms isolated from other species of animals.

In 1923 Spray isolated three groups of pasteurellae with a number of variants in each from an edematous type of pneumonia in spring lambs as found under slaughterhouse conditions. His Pasteurella ovisepticum, S-8 type, belonged among the classical

pasteurellae and would be included in Jones' Group III based on biochemical reactions. His second and third groups, isolated in pure culture, were separated from the true Pasteurella ovisepticum by their ability to hemolyze blood and ferment maltose and glycerol. The two types, S-3 and S-14, produced a distinct ring of partial hemolysis around the periphery of a rather flat, thin, translucent colony. Nitrates were reduced to nitrites, and indol was not produced. Action in litmus milk was neutral with reduction after prolonged incubation. Spray found both types highly pathogenic for mice and guinea pigs, but less so for rabbits. He considered them capable of causing a septicemia.

He separated these two hemolytic types from each other by three tests: (1) S-3 fermented lactose after seven or eight days. S-14 showed no action on lactose. (2) In 5 per cent glycerol-serum broth, S-3 fermented glycerol readily and produced a heavy, flocculent sediment which was not diffused by vigorous shaking. S-14 fermented glycerol after fourteen days and produced a moderate sediment which was not flocculent and diffused readily. The serum broth was made by adding an equal part of serum water (one part of serum to three parts of distilled water) to standard veal infusion broth, adjusted to pH 7.4, and containing 5 per cent glycerol and 1 per cent Andrade indicator. (3) Direct agglutination and absorption tests identified

two distinct groups coinciding with those indicated by the lactose, glycerol tests. No attempt was made to correlate these results with the findings of Jones

In England, Edington (1930) described an outbreak of pneumonia in seven cows and two calves caused by a hemolytic pasteurella. He found by agglutination and absorption tests that these organisms belong to Jones' Group I. He described the organism in more detail than did Jones and Spray. He observed that the organism showed a decided preference for moisture. When surface growth from a plate was collected in a heap, it had a yellowish tint with a slightly waxy consistency. In broth it grew readily producing first a turbidity and later a sediment. There was a tendency to pellicle formation. It grew on MacConkey's bile salt agar. He reported that in severe, rapidly fatal cases, as seen in the calves, the bacillus was cultured from the blood stream. One of the strains failed to hemolyze blood although all the other characteristics were the same.

In 1932, Newsom and Cross, working with strains of pasteurella, both hemolytic and nonhemolytic, from sheep and cattle affected with pneumonia, identified four serological groups. These authors proposed the name Pasteurella hemolytica for those strains showing hemolysis on rabbit blood agar, negative indol production, avirulence for rabbits, and acid from unheated dextrin, inositol, and maltose.

Bergey later adopted this name. Serologically, most of the hemolytic strains fell into one group which was identical with Jones' Group I. Two strains comprised the second group, but these were easily separated from the predominant group biochemically by their fermentation of mannose and inactivity on raffinose. Newsom and Cross tried to duplicate Spray's separation of the hemolytic strains by means of glycerol fermentation, but were unsuccessful since they found fermentation slow and uncertain.

Six strains of P. hemolytica were included by Rosenbusch and Merchant (1939) in a study of the hemorrhagic septicemia group. The biochemical results on the hemolytic strains, designated by them as Group IV, were identical with those of Newsom and Cross. They did not conduct serological tests on this group.

Routine diagnostic tests conducted on P. hemolytica cultures at Michigan State College revealed a disagreement with previous workers concerning the sugars fermented.[1] These differences are shown in Table I. Jones reported dextrose, lactose, maltose, mannite,

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[1] The sugars used routinely for identification of organisms are dextrose, lactose, maltose, mannite, and sucrose. These in conjunction with colony morphology, Gram stain, motility, indol production, and hydrogen sulfide production (Kligler Iron Agar) are usually considered sufficient to identify the typical pathogens encountered in poultry diseases.

TABLE I

COMPARISON OF FERMENTATION REACTIONS OBTAINED BY VARIOUS WORKERS

	Jones	Spray	Eding-ton	Newsom Cross	Mer-chant[1]	Bergey's Manual	M.S.C.
Dextrose	A	A	A	A	A	A	A
Lactose	AI	V	A	V	AI	V	A
Maltose	A	AI	A	AI	A	A	V
Mannitol	A	A	A	A	A	A	A
Sucrose	A	A	A	A	A	A	A
Dextrin		-	-	AI	A	A	V
Fructose		A	A	A	A	A	A
Galactose		A	A	V	A	A	V
Glycerol		AI	V	V	A	V	A
Inositol		-	V	AI	A	A	V
Raffinose			-	V	A	A	V
Sorbitol		A	A	A	A	A	A
Xylose		A	A	A	A	A	V
Arabinose		-	V	-	V	-	V
Dulcitol			-	-	V	-	-
Inulin		-	-	-	-	-	-
Mannose			V	V	V	-	V
Rhamnose				-	-	-	-
Salicin	-	-	-		-	-	-
Adonitol			-		-		-
Starch			-				V
Indol	-I	-	-	-I	-I	-I	-
Hemolysis	+I	+I	+[2]	+I	+	+I	+

A = acid produced; V = variable acid production; + = positive; - = negative, or no acid; I = considered an identifying characteristic.

[1] Taken from Veterinary Bacteriology and Virology.

[2] Some strains negative.

and sucrose consistently fermented and considered the slight and sometimes slow fermentation of lactose characteristic. Newsom and Cross reported lactose fermentation variable and considered maltose fermentation an identifying characteristic. In the poultry diagnostic laboratory, lactose fermentation, although slight, was consistently positive, while maltose fermentation was variable.

Because each worker used a different method for demonstrating acid production, a comparison of results was difficult. Jones recorded all reactions in terms of final pH, the lowest of which was 6.1. The pH produced in lactose broth by newly isolated strains was between 6.4 and 6.8. The method Jones used to determine pH was not given. By adding brom phenol blue, pH range 6.3 to 7.6, to fermented sugar broths at Michigan State College, the pH level given by Jones for lactose broth was duplicated. The pH in the other common sugar broths was below 6.3. The visible range of color transformation for brom cresol purple, which was used by Newsom and Cross, is 5.0 to 6.4. The yellow color, which is characteristic in the acid range, is barely visible through the purple at pH 5.8 and begins to predominate at pH 5.4. Considering these facts, the variability of lactose fermentation as recorded by Newsom and Cross was understandable. Andrade indicator, used routinely in the diagnostic laboratory, turns a definite pink at pH 6.8, and was used throughout these studies.

Variability of maltose fermentation, however, was not as easily explained since it was recorded while using a more sensitive indicator, and noted only in the poultry diagnostic laboratory. The possible existence of two serological strains of P. hemolytica separable by the fermentation of maltose was considered since Spray, and Newsom and Cross had apparently correlated serological properties with biochemical activity on carbohydrates. The thesis problem as originally approved, consisted of an attempt to demonstrate the presence of more than one serological type of P. hemolytica isolated from chickens, and to correlate such results with the fermentation of maltose and any other fermentable substance if such correlation proved possible.

Soon after the serological phase of the problem had been well established, a laboratory mishap resulted in the dissociation of the organisms under study. The distilled water supply became contaminated with tap water which was introduced through a leak in the still. Before the contamination was discovered, this water was used to prepare media on which the cultures for this project were transferred. Trace elements thus accidentally introduced into the media caused dissociation and rendered many strains inagglutinable, including those used in antisera production. By the time the source of the toxicity had been identified, most of the cultures transferred to

uncontaminated media prior to the mishap were no longer viable since cultures must be transferred once a week to insure survival. A continuation of the problem was not possible until a new collection of cultures could be made. It was decided at this time, in conference with the major professor, that a study of the dissociation pattern of P. hemolytica was essential to an understanding of the organism in view of its extreme sensitivity. One of the dissociated cultures and seventeen strains isolated following the mishap were examined for this purpose.

EXPERIMENTAL STUDIES

Preliminary--Selection of Media

From the first it was apparent that the organism under study was a fastidious one and that a medium for propagation must be selected with care. Berkman (1943) included one strain of P. hemolytica in a study of the accessory growth factor requirements of the genus Pasteurella and reported good growth of this species in his basal medium composed of hydrolyzed gelatin, inorganic salts, dextrose, and amino acids without the addition of accessory growth factors.

Procedure

Nine agar media without the addition of any growth factor were chosen for comparison. (1) Tryptose agar (Difco)[1] containing 2.0 per cent tryptose, 0.1 per cent dextrose, and 0.5 per cent sodium chloride, was chosen as a standard because it was known to be satisfactory for growing many fastidious organisms. Prepared according to directions, the resulting pH of the medium is 6.9. (2) Tryptose agar adjusted with sodium hydroxide to pH 7.2 was used to see what

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[1] Difco Laboratories Incorporated, Detroit 1, Michigan.

effect raising the pH might have. (3) Nutrient agar (Difco) containing 0.3 per cent beef extract and 0.5 per cent peptone, was chosen to represent a less nutritious medium. Prepared according to directions, the pH is 6.8. (4) Nutrient agar was also adjusted to pH 7.2. Darby's lactose motility medium, a modification of the Difco product, containing 1.8 per cent motility medium (Difco), 0.2 per cent beef extract, 1.0 per cent lactose and 1.0 per cent Andrade indicator, had been used routinely in the poultry diagnostic laboratory as a screening test to eliminate slow lactose fermenters and to classify lactose negative bacteria by motility. Since it had been observed that P. hemolytica grew well in this medium, two modifications of it were included in the survey: (5) motility medium prepared according to directions, containing 1.0 per cent tryptose and 0.5 per cent sodium chloride, plus enough agar to make 1.5 per cent; and (6) motility medium plus 0.2 per cent beef extract as in Darby's modification and agar to 1.5 per cent. Lactose was omitted since a production of acid in a medium for propagation was considered undesirable. (7) Brain heart infusion agar (Difco) containing infusion from 200 g of calf brains and 250 g of beef heart per liter, 1.0 per cent proteose peptone, 0.2 per cent dextrose, 0.5 per cent sodium chloride, and 0.25 per cent disodium hydrogen phosphate plus 1.5 per cent agar, was chosen because it is a much richer medium than tryptose. (8) Scott's

salts agar was included because Eveleth had used it with success for the maintenance of viability and pathogenicity of Pasteurella multocida. It was hoped that this medium would prove equally satisfactory for P. hemolytica. It contained 1.0 per cent peptone, 2.0 per cent agar, 0.2 per cent potassium citrate, 0.05 per cent potassium bicarbonate, 0.06 per cent ammonium dihydrogen phosphate, 0.25 per cent ferric ammonium citrate, and 0.05 per cent dextrose. (9) Chicken infusion agar containing the infusion from 500 g of ground chicken meat and bones per liter, plus 0.5 per cent sodium chloride, 1.0 per cent peptone, and 2.0 per cent agar, was tried to see if chicken proteins would prove satisfactory.

Criteria for judging the suitability of the media were rate of growth, amount and appearance of the growth present in 24 and 48 hours, sensitivity of the cells to physiological salt solution, morphology of the cells as determined by staining, and the viability of cultures after eight days, which was the longest period viability had been maintained at that time.

Duplicate slants of each medium were heavily streaked with 24-hour cultures of 772M and 309 and incubated at 37 C. Slants were examined at 4, 6, 8, and 24 hours for definite growth. At 24 hours, safranin stains were made from each medium showing a satisfactory amount of growth. Physiological saline was added to the

remaining growth and the turbidity adjusted to tube 2 MacFarland nephelometer. Ease of suspension and evidence of agglutination were noted. Suspensions were held and observed for four days. The duplicate culture in each case was reincubated another 24 hours and appearance of growth noted. The culture tubes were then sealed with parafilm and held at room temperature for six days. Transfers were then made to the same medium in each case.

Results

The significant information obtained from this study is recorded in Table II. Nutrient agar at both pH 6.8 and 7.2 and Scott's salts agar were discarded because of uniformly poor growth. Both of the motility medium modifications were eliminated as neither strain grew well on the basic motility medium, and strain 309 did not grow well on motility medium when beef extract was added. Although growth of strain 772M was good when beef extract was added, cells showed increased sensitivity to physiological saline. Brain heart infusion agar was likewise eliminated because organisms grown on it showed marked salt sensitivity. Chicken infusion agar supported luxuriant growth as did tryptose agar at both pH 6.9 and 7.2. Stained organisms from chicken infusion agar were uniform in size and shape at 24 hours. The size and shape of cells from

TABLE II

STUDIES OF GROWTH ON VARIOUS MEDIA

Agar Media	First Appearance of Growth Within 8 Hours		Amount of Growth				Growth on Transfer	
			24 Hours		48 Hours			
	772M	309	772M	309	772M	309	772M	309
1. Tryptose (pH 6.9)	6*	6	4+	4+	4+	4+	+	+
2. Tryptose (pH 7.2)	6	6	4+	4+	4+	4+	+	+
3. Nutrient (pH 6.8)	8	-	+	±	+	±	-	
4. Nutrient (pH 7.2)	8	-	+	±	+	±	+	
5. Motility	8	-	2+	+	2+	+	+	
6. Motility plus beef extract	8	8	4+	+	4+	+	-	
7. Brain-heart infusion	8	6	+	4+	4+	4+	-	-
8. Scotts	-	-	±	-	+	-		
9. Chicken infusion .	-	6	4+	4+	4+	4+	-	-

* = hours; + = growth visible; 2+ = growth moderate; 4+ = maximum growth.

TABLE II (Continued)

Saline Suspensions	Morphology of Cells in 24 Hours	
	772M	309
HS	Mostly disintegrated--remaining cells coccoid.	Coccobacilli, few bipolar
HS	Same as medium 1	Slender bacilli, few filaments
	Mostly coccobacilli--few long filaments	
	Coccobacilli	
	Irregular in staining--"shadow cells"	"Shadow" and involution forms
PA	Slender bacilli pale staining	Coccobacilli, few involution forms
A	Coccoid forms in clumps	Size irregular
HS	Slender bacilli	Coccobacilli

HS = homogeneous, stable suspension; PA = partially agglutinated, settled out in four days; A = strong agglutination.

strain 309 when grown on tryptose agar were uniform at 24 hours, but those of strain 772M showed mostly disintegrated cells. Cells from all media stained at 48 hours showed the same disintegration, often with poorly stained "shadow cells" and involution forms appearing before complete disintegration. This process suggests the presence of a very active autolytic enzyme. Viability was not maintained on chicken infusion agar, however. Subcultures made on the eighth day did not grow. Tryptose agar was the only medium which supported satisfactory growth of both strains. It also maintained viability for the longest period of time and was selected for propagation of P. hemolytica.

When the studies on dissociation were started, it was discovered that although the newly isolated strains appeared to grow satisfactorily on tryptose agar slants, cell suspensions plated for isolated colony study on tryptose agar plates did not produce the expected number of colonies as previously determined by plating dilutions of strains of 309 and 772M. The same cell dilution plated on tryptose blood agar base without blood yielded the expected number of colonies. Tryptose blood agar base contains 1.0 per cent tryptose, 0.3 per cent beef extract, and 0.5 per cent sodium chloride, and is similar to trial medium number 6. All the strains used for dissociation studies produced a larger number of colonies on tryptose blood agar

base than on tryptose agar except strains 309 and 328, which grew more satisfactorily on tryptose agar. The medium which best supported the growth of the strain being examined was used throughout the dissociation studies.

Serological Relationships

An attempt was made to separate the strains of P. hemolytica serologically using a maltose fermenting strain and a maltose negative strain. Strain 309 was selected to represent the maltose negative strains. At the beginning of this phase of the work, strain 309 had been freshly isolated in almost pure culture from a bird showing hyperemia and patchy consolidation of the left lung as the only pathological change. Ten per cent of the flock of 3,500 were visibly ill. A 6 per cent loss was reported in a three-day period. Strain 772M was selected to represent the maltose positive strains. The letter M after any culture number indicates that the culture fermented maltose. 772M was isolated six months before strain 309 from a bird affected with the visceral form of the avian leucosis complex. In addition to the neoplastic changes in the spleen and kidney, the liver of this bird was ochre in color with necrotic foci, one kidney was cystic, the ova discolored, and an abscess was noted in the cecum. A moderate growth of P. hemolytica was obtained.

No other bacterium of significance was isolated. This strain was chosen chiefly because a maltose negative strain was also isolated from the same bird.

Both 309 and 772M were stable in physiological saline. In addition to the maltose variation, these two strains also differed in opacity of growth. The growth of strain 309 was almost transparent on a tryptose slant like that of a streptococcus, while that of 772M was more translucent. The majority of strains isolated were translucent and like 772M in appearance. Strains 772, maltose negative, 1138 and 1304 were less opaque than 772M. Strains 767, 818M, and 955 were more opaque and mucoid.

Procedure

Each of two adult rabbits per strain was given an intraperitoneal injection of 0.5 ml of a 24-hour live, saline suspension of organisms. A turbidity corresponding to tube 1 MacFarland nephelometer was used. A second intraperitoneal injection of 1.0 ml was given on the third day. All injections were made at three-day intervals. The third, fourth, and fifth inocula were 0.5 ml introduced intravenously. The sixth, seventh, and eighth inocula were 1.0 ml given intravenously. The ninth and all subsequent injections were

1.0 ml of a live, saline suspension with turbidity corresponding to tube 2 MacFarland nephelometer introduced intravenously.

One month after the first inoculation, blood was drawn from the rabbits and agglutination tests conducted. The antigen used was a 0.5 per cent phenolized saline suspension with a turbidity corresponding to tube 1 MacFarland nephelometer. Tests were incubated for 18 to 20 hours at 37 C. Serum from all four rabbits showed a weak agglutination with homologous serum through a dilution of 1-40. Injections were continued every three days for two additional months. The titers of sera pooled from the two rabbits receiving the same inoculum were 20,000 and 10,000 respectively for 772M and 309.

Agglutination tests were conducted using five strains of P. hemolytica against each antiserum. Before adsorptions and further agglutinations were completed, the twenty-two cultures being studied were accidentally dissociated as previously described.

Results

Table III shows the results of the agglutinations that were completed. Although some of the tubes showed clearing with complete precipitation of antigen, the clumps were small when the reactions were shaken. When 772M was used as antigen against antiserum 309, agglutination occurred through a serum dilution of 1-50 in contrast

TABLE III

AGGLUTINATION REACTIONS OF SEVERAL STRAINS OF PASTEURELLA HEMOLYTICA AGAINST ANTISERA PREPARED FROM STRAINS 309 AND 772M

Antigen	Serum	Serum Absorbed With	Titer	Agglutination Complete Through Dilution [1]	Controls [2]
772M	772M		1,600	1-200	-
309	772M		0	-	-
309	309		3,200	1-800	-
772M	309		50	Inc.	-
772	772M		0	-	-
772	309		6,400	1-400	-
818M	772M		0	-	-
818M	309		400	1-100	-
818	772M		100	Inc.	+
818	309		1,600	Inc.	+
784	772M		0	-	-
784	309		0	-	-
767	772M		3,200	Inc.	-
767	309		1,600	1-800	-
309	309	772M	3,200	1-800	-

Inc. = incomplete clearing of supernatant fluid with definite but incomplete agglutination; - = negative agglutination; + = positive agglutination.

[1] Complete clearing of supernatant fluid with complete agglutination.

[2] Both antigen and negative serum controls used.

to the 1-3,200 recorded when homologous antigen was used. No agglutination occurred when 309 was used against 772M antiserum, however. It is interesting that antigen 772 was not agglutinated by antiserum 772M although both cultures were present in the same bird, but was agglutinated by antiserum 309. Neither 772 nor 309 fermented maltose. Culture 818 apparently was rough since the antigen control showed slight agglutination following incubation. Culture 767 was agglutinated by both antisera although the reactions were more complete with antiserum 309. Culture 818M agglutinated with antiserum 309 only.

Fermentation Reactions

Procedure

The base medium used in studying the fermentative ability of P. hemolytica consisted of 1.0 per cent tryptose, 0.5 per cent sodium chloride, 1.0 per cent Andrade indicator, and 0.2 per cent agar. Growth was more uniform in this semisolid medium than in the usual broth medium. This base was autoclaved at 15 pounds steam pressure for 20 minutes. One per cent of the desired sugar was added to the base, and the medium was resterilized at 12 pounds pressure for 20 minutes.

The twenty-two strains used in this study were not inoculated into all the sugars immediately upon isolation, but were carried on tryptose agar until a number of strains were available. Inoculations were then made at one time. Each sugar was heavily seeded with a loopful of 24-hour tryptose broth culture and observed daily for two weeks. The cultures were transferred once a week for several months and reinoculated into sugars for comparison with the first set of results.

Results

Acid was produced from dextrose, maltose, mannitol, sucrose, galactose, xylose, mannose, and trehalose in 24 hours. Lactose was positive in 24 or 48 hours, but the acidity produced was slight. A small amount of acid was produced from sorbitol in 48 hours; this reaction faded in four days and appeared negative thereafter. Glycerol and raffinose were fermented in three to six days. Production of acid from dextrin and starch took five to seven days in the few instances that these substances were fermented. Table IV shows the substances fermented.

It was observed that two of the maltose-fermenting strains lost the ability to ferment several of the sugars after several months. Strain 772M failed to ferment sorbitol on the second study. This was

TABLE IV

FERMENTATION REACTIONS OF TWENTY-TWO STRAINS OF PASTEURELLA HEMOLYTICA ISOLATED FROM CHICKENS

	Dextrose	Lactose	Maltose	Mannitol	Sucrose	Glycerol	Raffinose	Mannose	Galactose
309	A	A	-	A	A	A	S	A	A
767	A	A	-	A	A	A	S	A	A
772	A	A	-	A	A	S	S	A	A
784	A	A	-	A	A	S	S	A	-
818	A	A	-	A	A	A	S	A	A
955	A	A	-	A	A	A	S	A	A
1138	A	A	-	A	A	S	S	A	A
1304	A	A	-	A	A	S	S	A	A
75	A	A	-	A	A	S	S	A	A
698	A	A	-	A	A	S	S	A	A
540	A	A	-	A	A	S	S	A	A
601	A	A	-	A	A	S	S	A	A
579	A	A	-	A	A	S	S	A	A
556	A	A	-	A	A	A	S	A	A
541	A	A	-	A	A	S	S	A	A
94	A	A	-	A	A	S	S	A	A
76	A	A	-	A	A	S	S	A	A
699	A	A	-	A	A	S	S	A	A
772M	A	A	A	A	A	A	S	A	A
818M	A	A	A	A	A	S	S'	A'	A'
540M	A	A	A	A	A	S	S	A	A
541M	A	A	S	A	A	S	S	A	A
3377M*	A	A	A	-	A	A	S	A	A
3382M*	A	A	A	-	A	A	S	A	A

A = acid produced within two days; S = acid produced in three to seven days; - = no acid; ' = lost ability to ferment after several transfers; * = porcine strains.

TABLE IV (Continued)

Sor-bitol	Xy-lose	Tre-halose	Dex-trin	Starch	Arab-inose	Dul-citol	In-ulin	Rham-nose	Sal-icin	Adon-itol
A	-	-	-	-	-	-	-	-	-	-
A	A	A	-	-	-	-	-	-	-	-
A	A	S	-	-	-	-	-	-	-	-
A	A	A	-	-	-	-	-	-	-	-
A	A	A	-	-	-	-	-	-	-	-
A	A	S	-	-	-	-	-	-	-	-
A	A	A	-	-	-	-	-	-	-	-
A	A	A	-	-	-	-	-	-	-	-
A	A	A	-	-	-	-	-	-	-	-
A	A	A	-	-	-	-	-	-	-	-
A	A	A	-	-	-	-	-	-	-	-
A	A	A	-	-	-	-	-	-	-	-
A	A	A	-	-	-	-	-	-	-	-
A	-	S	-	-	-	-	-	-	-	-
A	A	A	-	-	-	-	-	-	-	-
A	A	A	-	-	-	-	-	-	-	-
A	A	A	-	-	-	-	-	-	-	-
A	A	A	-	-	-	-	-	-	-	-
A'	A	S	S	S	-	-	-	-	-	-
-	A'	A	-	-	-	-	-	-	-	-
-	A	A	S	S	-	-	-	-	-	-
-	A	A	S	S	S	-	-	-	-	-
A	A	A	S	S	S	-	-	-	-	-
A	A	A	S	S	S	-	-	-	-	-

of interest since all the other maltose positive strains failed to ferment sorbitol at any time. It was noted during the subsequent work on dissociation that all newly isolated strains fermented sorbitol slightly. Strain 818M failed to ferment raffinose, galactose, xylose, and mannose after a period of artificial cultivation. Occasionally a strain failed to attack a substance utilized by the majority of strains, but no pattern was observed. Strain 309 was of interest because it fermented fewer sugars than any other strain.

Cultures 3377M and 3382M were obtained from Dr. John Collier at Iowa State College. They were isolated from lung lesions in pigs and are included for comparison with the strains isolated from chickens. These two strains were not used in any other studies for this thesis because they agglutinated rapidly in 1-500 acriflavin. The strains used by Jones and others were not available.

Dissociation Patterns

The term "dissociation" is used to denote one of several types of variation which occur in bacteria. It refers specifically to changes in colonial morphology from smooth (S) or mucoid (M) to intermediate (I) or rough (R) and other altered characteristics which may be associated or appear independently. The typical smooth (S) colony is round with an entire margin, soft in texture and glistening

in appearance. It is composed of cells which show little variation in size or shape, make homogeneous suspensions in saline and agglutinate readily in specific serum. The rough (R) colony is larger and flatter with a rough or corrugated surface, not soft and often dull in appearance. It is composed of cells which are longer, pleomorphic and agglutinate spontaneously in saline. Mucoid (M) types take their name from their mucoid texture which is dependent on a well-developed capsule. It may represent the virulent phase of the organism. Intermediate (I) types are usually less stable than S, R, or M, and represent stages between these phases.

Braun (1947) states:

> . . . the phenomenon of dissociation appears to resolve itself into merely another manifestation of causally related mechanisms of discontinuous bacterial variation which involve the spontaneous occurrence of undirected variants (mutants) and their subsequent establishment within a population under the control of inherent and environmental factors governing population dynamics.

Braun further states that the bulk of the evidence available supports the mutation concept of dissociation. The occurrence of mutants in bacteria has been established by Zelle and others as comparable in rate to those of higher organisms. The inherent factors involved in the establishment of a mutant are the genetic ratio, the rate with which stable cells split off from unstable cells, and the relative growth rate of the two types in the environment being used. When

the environment is less than optimum, a high differential between the total number of cells present in the medium and the number of viable cells leads to population pressure which permits the selection and establishment of any mutant with a higher growth rate or viability. It has been proved with Brucella abortus that an ideal medium produces a low differential with little or no population pressure resulting in the reduction or absence of dissociation. In some cases the addition of 2.0 per cent normal serum to a medium may completely inhibit dissociation.

Since mutation may take place at different levels, variations may occur independently or in combination. Changes in biochemical activity and cell morphology often take place independently, but the loss of polysaccharide surface antigen is usually responsible for loss of type specific antigenicity, increased agglutinability by salt, altered sensitivity to bacteriophage and loss of virulence. Many important exceptions to these general rules are known.

The following characteristics were chosen for this study on the dissociation of P. hemolytica: (1) colonial changes, (2) sensitivity to sodium chloride as evidenced by growth characteristics in broth, stability of organisms in saline suspensions at room temperature and 80 C, sensitivity in the presence of acid and agglutination by acriflavin, (3) presence of capsules, (4) variation in morphology of cells,

and (5) variation in biochemical activity. Virulence studies were not included because the organism is normally a weak pathogen, and housing facilities for the number of chickens required for statistical data were not available at the time.

Colonial Changes Occurring During Dissociation

The phases present in culture 309 following accidental dissociation and those arising during the study were described first. Then as new cultures were isolated, the phases present at the time of isolation were described. It was hoped that S and R types would be identified.

Procedure. Both tryptose agar and tryptose agar base were used. The medium which produced the larger number of colonies from a given dilution of that culture was selected. The agar content was adjusted to 2.0 per cent so that a firm surface was obtained for streaking cultures with a wire loop. Agar plates were poured to a thickness of one-quarter inch and incubated 24 hours to dry the surface before use.

No single procedure of seeding cultures on the plates to obtain adjacent and contiguous colonies proved superior. Two methods were used. The least time-consuming was direct streaking of a

small inoculum with a wire loop over the entire surface of a plate. This produced confluent growth, contiguous, adjacent, and well-isolated colonies provided the amount of inoculum was carefully controlled. A colony selected for study was picked by use of the stereoscopic microscope using a fine, flexible, one-inch wire which had been removed from a light bulb, flattened on one end, and inserted into an inoculating-needle holder. The amount of inoculum transferred to the plate was just barely visible to the unaided eye. The inoculum was then streaked over the entire surface of the plate using a standard-type loop. This was satisfactory when single phases were streaked for descriptive purposes. Since coloring of colonies is dependent on size and proximity of the colonies, this procedure allowed one to become familiar with the variations of each phase. The method was also satisfactory for streaking two phases together for comparison or verifying that they were in the same phase. Contiguous colonies are best suited for this purpose. The other method consisted of diluting the culture in saline to such a concentration that 1.0 ml of suspension distributed over the plate would produce sufficiently isolated colonies. The advantage here lay in greater uniformity of coloring which resulted in greater ease of examination. On the other hand, closely related phases are often indistinguishable unless the colonies are contiguous, and the uniform distribution expected by this

method usually produced few such colonies. Dilution of the broth culture for optimum concentration of colonies is dependent on colony size, strain, and phase. Culture 309 was diluted 1-500,000 while culture 684, phase B, required dilution to 1-4,000,000 for satisfactory distribution. Forty-eight-hour broth cultures were used since 24-hour cultures gave variable results. This method was very time-consuming. A colony picked from a plate was placed on a tryptose agar slant, incubated for 24 hours, then transferred to tryptose broth, incubated for 48 hours, and diluted in saline for inoculation onto plates. All plates were incubated 48 hours before examination. Thus, five days were required to obtain the same information obtainable in two days by direct streaking. This method had the further disadvantage that cultures dissociate more rapidly in liquid media than in solid.

Cultures were examined using a stereoscopic microscope at magnifications of 9X and 18X. The source of illumination was an American Optical Spencer microscope illuminator No. 735B set on a block 4-1/2 inches high and placed at a distance of 10-3/4 inches from the microscope. The adjustable condensing lens of the lamp was so positioned as to produce maximum color differential in the bacterial colonies being examined. The lamp was tilted until the outside rim holding the filter in place rested on the front edge of

the supporting block. The diaphragm was opened as far as possible and the blue glass filter removed. A thin photographic plate with the emulsion removed was substituted for the glass stage of the microscope so that no light rays would be absorbed. A five-centimeter concave mirror was placed about 8-3/4 inches from the supporting block for maximum brightness. The angle of reflection was approximately 45°.

Results. All colonies from cultures 309, 710, 684, 782, 868, and 328 were circular, glistening, and ranged from 1.0 mm to 2.5 mm in diameter. Most colonies were convex and butyrous. The opacity of all 309 phases was grossly much less than that of the other cultures except 684 A, Av1, Av2, and 328, which were more opaque than the 309 phases when viewed under the microscope. Differences in opaqueness of other phases were not visible to the unaided eye.

Culture 309 was studied over a period of four months. There were three phases present when first examined: Y, YO, and some striated colonies, St. The striated colonies, when restreaked, yielded St colonies plus a third stable phase, YT. Phase YT occasionally produced St colonies which were at first thought to be a transition stage between YT and another phase. No phase arose from either

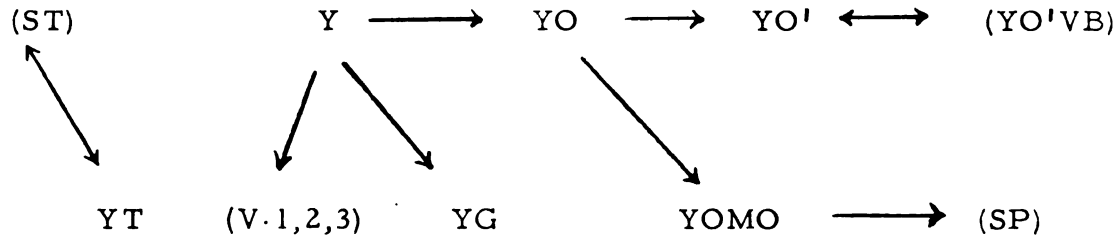
St or YT during the period of study, however. Other stable and unstable phases arose from Y and YO in sequence as shown in Plate I. Descriptions of the stable phases appear in Table V.

Other rapidly changing phases were also found. A phase designated YO'vb was occasionally isolated from YO'. It was 1 mm in size, pulvinate, mucoid, and cottony in appearance under the microscope. The color was yellow buff, and the colony was more opaque than YO' and more granular than any of the stable phases. When restreaked, it gave rise to YO'vb and YO' colonies plus some light unorganized, mush-appearing growth in the background. If held on slants and transferred several times, it reverted to YO'. Morphology of cells did not differ from YO'.

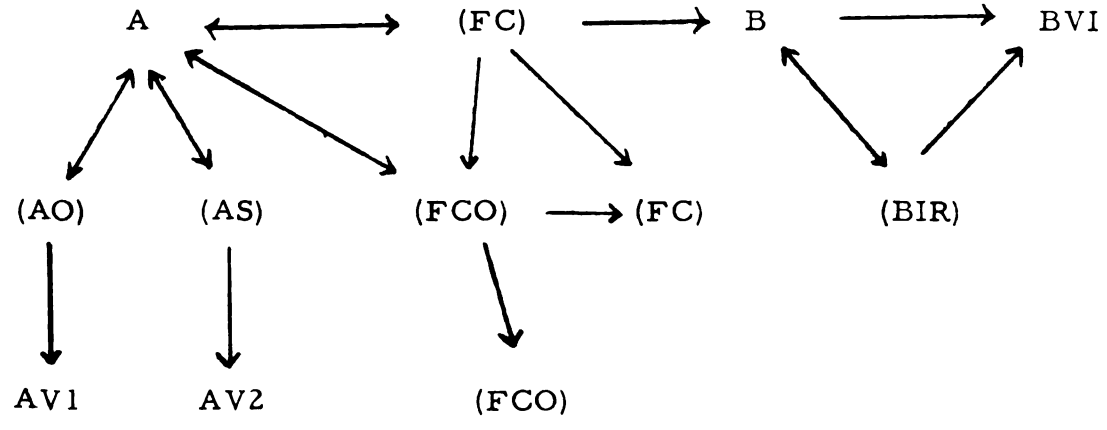
Another group of transitory colony types, designated the V group, originated from phase Y. All these types had an irregular margin. The first to appear was a flat, translucent colony with a bright refractive margin. The cells from this colony when stained were found to consist of long curved filaments which may be seen in Plate VI, Figure 4. This colony, when transferred to another plate, broke down into three related types: V-1 was a clear, gray, evenly granular colony with a very irregular margin. This colony when streaked produced like colonies which did not grow on transfer. V-2 was similar to V-1 except for a heavy, prismatic strip appearing

PLATE I

DISSOCIATIVE PATTERNS



Culture 309



Culture 684

( ) = unstable phases.

TABLE V

COMPARISON OF PHASES: CULTURE 309

Phase	Colony Appearance	Opacity	Coloring
Y	1.5 mm, convex, entire, butyrous, granules fine.	L YO* G YO'*	Center--more opaque, yellow buff; margin--thinner with blue to red rainbow effect; confluent colonies appear pink; red predominates more than in YO or YO'.
YO	1.5 mm convex, entire, butyrous, granules finer than Y.	G Y, YO'	Center--warm cream; margin --clear, narrow, and bluish; little red in coloring; yellows predominate.
YO'	1.5 mm, convex, entire, butyrous, granules finest.	least opaque	Rainbow effect throughout colony from blue on bottom to yellow to red on top; blue-greens predominate.
YT	1.5 mm, convex, entire, butyrous, granules as YO.	G YO' L YO	Center--orange yellow for 1/2 area; margin--green-blue; some red on top; closer in hue to YO' than YO.
YOMO	1.0 mm, entire colony lifts from agar when touched, granules as YO.	most opaque 309	Cream white.
YG	1.5 mm, convex, entire, butyrous, granules as Y.	same as Y	Appears like Y in texture, but green-yellows predominate; hue closest to YO'.

* L = less than; G = greater than.

along one side. When transferred to another plate, this colony produced V-2 and V-3 type colonies, but when the resultant V-2 was streaked, the colonies were small, gray, flat, and did not grow when subcultured. The V-3 colony was entirely prismatic and more opaque than V-1. When streaked on another plate two colony types appeared which did not grow on transfer. One was V-3 type; the other was more opalescent with a blue edge. The surface was smooth in reflected light but appeared to have eccentric lines when viewed with transmitted light.

A colony similar in appearance to the original V-type colony developed from phase YOMO. It was streaked on a fresh plate to see if V-1, 2, or 3 would be produced. After two transfers the crystalline edge disappeared. On the following transfer three phases were present. One seemed to be the same as the previous type. Both other types were larger than the stable 309 phases and spreading in nature. Sp-1 was a flat, orange colony with an irregular edge. Sp-2 was raised, more buff colored, and smooth with a thin edge. Transfers from these colonies did not grow. The six stable phases were lyophilized for reference using sterile milk as the vehicle.

Culture 684 was isolated on blood agar from the liver of a chicken affected with Newcastle disease. Two colonies were transferred from the blood plate to lactose motility tubes and then streaked

on tryptose agar plates. Growth was not satisfactory as previously indicated, and tryptose blood agar base was substituted. Four colony types were present: A, B, FC, and FCO. Stable colony types present at the time of isolation were designated by letter (A, B, etc.), and stable phases arising from them later were designated by the phase letter from which the new phase arose plus v (variant) and numbers to differentiate these new variants. (When two phases were found to develop from 684 A, they were labeled Av1 and Av2.) All new colony types were streaked with all previously described stable phases, and if they were in the same phase, were given the same identification. All results were checked many times. Descriptions of phases A and B together with the other stable phases arising from them appear in Table VI. Sequence of appearance of variants appears in Plate I. FC and FCO were transitory phases originating from A. FC was 1.0 to 1.5 mm in diameter, flat, gray, granular, and nearly transparent. When transferred to another plate, phases FC, FCO, and B resulted. FCO appeared the same as FC except for a raised, opaque area varying in size and yellow cream in color. It gave rise to FC, FCO, and A colonies.

Two other transition colonies arose from A. AO was the same size as A but more opaque and buff in color. When streaked, A and Av1 colonies were present. Phase AS was half the size of

TABLE VI

COMPARISON OF PHASES: CULTURE 684

Phase	Colony Appearance	Opacity	Coloring
A	1.0-1.5 mm, convex, sometimes umbonate, entire, butyrous granules fine.	G any* 309	Buff yellow with orange center, some red on top edge, blue on bottom; when plated with B is smaller than typical colony and loses central raised area.
Av1	1.0-1.5 mm, convex, entire, butyrous, granules fine.	G A	Color and density more homogeneous than in A; yellow-orange throughout.
Av2	1.5-2.0 mm, convex, slightly irregular, butyrous, cottony texture.	L Av1	Color and density more homogeneous than in A; color paler than A, cream yellow.
B	2.0-2.5 mm, raised, entire, butyrous, granules fine.	G A	Color much like A; B gives appearance of being more orange when viewed alone, probably due to size; red fringe on top edge pronounced.
Bv1	2.0-2.5 mm, raised, entire, butyrous, granules fine.	same as B	Homogeneous pink-cream with faint yellow-green margin; when colonies well separated, looks like B; when streaked with B, looks pale, chalky, and more opaque.

* L = less than; G = greater than.

A, more opaque and buff in color. It produced A and Av2 colonies. Av1 and Av2 phases were also found without appearance of the transition phases. These stable phases may be seen in Plates II and IV.

Phase BIR was first seen on a saline dilution plate that was made from a broth culture of phase B that had been exposed to garlic vapors during incubation. It was identical to phase B except for the irregularity of the margin. This is shown in Plate IV, Figure 3.

Culture 782 was isolated from a bird submitted as a pullorum reactor. The bird gave a positive whole blood agglutination in the laboratory with polyvalent Redigen, but Salmonella pullorum was not isolated. Two phases were present which proved to be indistinguishable from 684 A and B, and were so designated 782 A and B. Stock cultures of these phases were being incubated the two succeeding week ends when garlic antibiotic cultures were placed in the incubator without permission of the persons using the incubator. Garlic appeared to have a marked antibiotic effect on P. hemolytica. Most of the dilution plates incubated at this time were sterile; the others had only a few, abnormally small colonies present. Agar slants of phases 782 A and B grew very poorly. When these cultures were plated following the first exposure to garlic vapors, the colonies

produced were atypical, and bacterial cells from them were extremely pleomorphic. After several subcultures in a normal atmosphere, the phases again appeared typical. Following the second exposure, however, two phases differing from A and B were found. The new phases were called C and H. Descriptions of these colonies are recorded in Table VII. Later another phase originated from C and was designated Cv1. None of these phases resulting from exposure to garlic vapors coincided with any of those formed without induced dissociation from 684 A and B.

Culture 868 was isolated from rachitic five-day old chicks. At first only one phase was thought to be present, but after several platings two phases called E and F were separated. These were differentiated chiefly by opacity. Phase E was found to vary in size from pin point to 1.5 mm with regularity, while phase F colonies ranged from 1.5 to 2.0 mm and had a slightly irregular margin. Neither phase corresponded with any phase previously described. A third phase arising from E was found to be identical in colonial appearance with 782 C and was designated 868 C. A description of these phases appears in Table VII. Pictures of phases E and F may be seen in Plates III and IV.

Culture 710 was isolated from a chicken affected with visceral lymphomatosis. Two colony types were present and are described in

TABLE VII

COMPARISON OF PHASES: CULTURES 782 AND 868

Phase	Colony Appearance	Opacity	Coloring
782 A	indistinguishable from 684 A		
782 B	indistinguishable from 684 B		
782 C	2.0 - 2.5 mm, raised, entire, butyrous, granules fine.	slightly L 684 B	Buff-yellow orange; hue closest to 684 B, but slightly more blue-green present.
782 Cv1	2.0 - 2.5 mm, raised, entire, butyrous, granules fine.	same as 684 B G 782 C	Homogeneous yellow cream with some blue present.
782 H	2.0 mm, convex, entire, butyrous, granules fine.	grossly L 782 C	Ivory yellow with blue-green margin which tapers out thin if colonies are well separated.
868 E	size varies from pin point to 1.5 mm, convex, entire, butyrous, granules fine.	G 782 C L 782 Cv1	Ivory buff with some blue tinge; coloring closest to 782 H.
868 F	1.5 - 2.0 mm, convex, slightly irregular, butyrous, cottony texture.	L 868 E same as 684 Av2	Pale ivory-yellow with blue-green tinge; very close to 868 E in hue, color more definite than in 684 Av2.
868 C	indistinguishable from 782 C		

L = less than; G = greater than.

Table VIII. Both of these phases agglutinated spontaneously in physiological saline. They were considered rough, and were designated R1 and R2. The morphology and growth in broth were described, capsule stains made, and the phases inoculated into carbohydrates. They were transferred to brain-heart infusion (Difco) with 0.2 per cent agar and stored at -40 C. It was hoped that they would remain inactive yet viable at this temperature until similar phases could be isolated from other cultures for comparison. The frozen preparations did not grow when thawed one month later. Other cultures did not change appreciably when stored in this manner up to three months.

None of the phases thus far described was found to be smooth when studied further. Colonies were picked from twelve other cultures upon isolation. None of these were smooth either. Two of the twelve cultures agglutinated spontaneously in 0.85 per cent saline. The remaining ten cultures agglutinated in acriflavin; three were positive in the rapid slide test, and the other seven were positive in the six-hour tube agglutination.

Finally, one phase which was considered smooth was isolated. The growth was quite transparent, the colonies were small, and resembled most closely those of culture 309. Growth on plates was very poor and isolated colonies rarely developed. The presence of blood markedly enhanced growth. Tryptose agar proved to be the

TABLE VIII

COMPARISON OF PHASES: CULTURES 710 AND 328

Phase	Colony Appearance	Opacity	Coloring
710 R1	2.0 - 2.5 mm, convex, slightly irregular, butyrous, granules fine.	L 684 B	Pale blue-yellow; concentric rings appear in transmitted light; surface smooth.
710	1.0 - 1.5 mm, flat, entire, brittle, powdered glass texture.	iridescent	All colors reflected by the particles; appears very bright.
328 S	1.0 mm, mucoid, slightly irregular, granules fine, like 309 Y.	G 309 Y	Top half pink, bottom half yellow; coloring brighter than 309Y; margin - very thin, narrow, clear.
328 I	1.0 mm, slightly irregular, brittle, powdered glass texture, slightly more granular than 328 S.	slightly L 328 S	Light pink throughout; yellow and red reflected from granules; margin almost prismatic.

L = less than; G = greater than.

most satisfactory medium without blood, but dissociation was rapid. Descriptions of the smooth phase, S, and another phase, I, appear in Table VIII.

An attempt was made to utilize 2,3,5 triphenyl tetrazolium chloride for greater differentiation of phases. It proved to be markedly inhibitory even at a concentration of 0.00125 per cent. The recommended concentration for distinctive colony coloring is 0.005 to 0.01 per cent.

Sensitivity to Sodium Chloride

Since the first studies on dissociation, auto-agglutination in physiological saline has been accepted as a means of confirming the roughness of a colony. At present, the phenomenon is generally regarded as the result of an increase in lipoidal substance on the surface of the bacteria due to the loss of normal polysaccharide antigen. The intermediate phases between S and R become increasingly sensitive to sodium chloride as more of this lipoidal substance is exposed. Intermediate phases which are stable in physiological saline at room temperature may often be detected by auto-agglutination at 80 C or in the presence of acid. The granular appearance of rough variants in broth cultures is attributed to the same phenomenon. The acriflavin test, which was first used by Pampana, is

believed by Edwards and Bruner (1942) to follow more closely the serological behavior of S and R antigens than any other indicator of roughness. Smooth phases are not agglutinated in acriflavine solution.

Growth characteristics in broth. All stable phases described were grown at 37 C in tryptose broth and examined daily for six days. Most phases produced a light uniform turbidity with some sediment and a ring on the surface of the broth in 48 hours. A partial pellicle formed in three to six days. No sediment or pellicle was formed in the broth inoculated with 328 S, the one smooth phase. Increased granularity was not noted grossly in the rough phases 710 R1 and R2, but the tendency to pellicle formation was more pronounced, and stains made from broth cultures revealed the presence of clumps.

Stability of organisms in saline suspensions. The growth of a 24-hour tryptose agar slant was suspended in sterile, 0.85 per cent sodium chloride and the turbidity adjusted to tube 1 MacFarland nephelometer. One ml of the suspension was pipetted into a 12 by 75 mm agglutination tube and incubated in a water bath at 80 C for one hour. Five ml of suspension were placed in a 16 by 150 mm test tube and examined daily for one week for evidence of agglutination.

None of the phases except 710 R1 and R2 showed evidence of agglutination. The rate at which suspended cells settled to the bottom of the test tube varied, however. Cells from phases 684 Av2 and Bv1 were partially settled within 24 hours. Phase 782 H was completely settled, and the 309 phases and 868 E were partially settled in one week. Cells from phases 684 A, Av1, and B, 782 C and Cv1, and 868 F were still in suspension after one week. None of the phases stable in saline at room temperature agglutinated at 80 C.

Sensitivity in the presence of acid. All phases studied were suspended at a turbidity corresponding to tube 1 MacFarland nephelometer in 5.0 ml of sterile, 0.85 per cent sodium chloride buffered at pH levels of 3, 4, 5, 6, 7, 8, 9, and 10 with "pHydrion Buffers" manufactured by the Micro Essential Laboratory, Brooklyn 10, New York. Suspensions were held at room temperature and examined in 24 hours and again after one week.

Results are tabulated in Table IX. Phases 309 Y, YG, and 328 were stable in saline at all pH levels. Most phases agglutinated at pH 3 after one week although phases 309 YO, YOMO, and 782 C were stable in 24 hours. Fourteen of the eighteen phases showed agglutination at pH 4, but the size of the agglutinated particles varied.

TABLE IX

AGGLUTINATION OF SALINE SUSPENSIONS PREPARED FROM STABLE PHASES UNDER VARYING CONDITIONS

Phase	0.85% Saline	Buffered Saline*		Acriflavin	
		24 Hrs. (pH)	1 Week (pH)	Plate	Tube
684 A	-	3	3, 4	-	+
684 Av1	-	3, 4, 5	3, 4, 5	+	+
684 Av2	-	3, 4	3, 4	+	+
684 B	-	3, 4'	3, 4, 5	-	+
684 Bv1	-	3, 4	3, 4	-	+
710 R1	+				
710 R2	+				
782 C	-	-	3, 4	+	+
782 Cv1	-	3, 4	3, 4	+	+
782 H	-	3, 4	3, 4, 5	+	+
868 E	-	3, 4, 5	3, 4, 5	+	+
868 C	-	3'	3	+	+
868 F	-	3, 4	3, 4	+	+
309 YT	-	3, 4	3, 4	-	+
309 YO	-	-	3	+	+
309 YO'	-	3, 4	3, 4	+	+
309 Y	-	-	-	+	+
309 YG	-	-	-	+	+
309 YOMO	-	-	3, 4	+	+
328 S	-	-	-	-	-

* = Phase showed agglutination with settling of particles at pH level indicated.

' = Agglutinated particles remained in suspension.

_ = Agglutination weak, particles small.

\+ = positive agglutination

\- = negative agglutination

Four of the eighteen phases agglutinated slightly at pH 5. No agglutination was noted above pH 5. In most tubes above pH 5 the cells had settled after one week. Maximum stability was observed at pH 10 although all phases stayed in suspension at pH 8 for 24 hours.

Agglutination by acriflavine. Both slide and tube methods were used. In the slide test, one loopful of heavy saline suspension of organisms was mixed with one loopful of 1-500 acriflavine in 0.85 per cent saline and watched for two or three minutes for agglutination. For the tube test equal parts (0.5 ml) of 1-1,000 acriflavine in saline, and suspended organisms of turbidity corresponding to tube 1 MacFarland nephelometer were incubated at 37 C for six hours.

The tube method produced more positive agglutinations than the slide method. Results are recorded in Table IX. No phases were studied which did not agglutinate in acriflavine except 328 S. Phases 684 A, B, Bv1, and 309 YT were positive in the tube test only.

Presence of Capsules

The method employed for demonstration of capsules is a modification of one described by Huddleson (1940) as recorded in Stafseth's laboratory manual (1953). Organisms were grown on

blood agar for at least two transfers and cells from a 24-hour culture suspended in a small drop of water using an inoculating loop. The drop was then smeared on the slide with a second slide as if preparing a blood film. When dry, this film was covered with a smear of India ink made in the same manner and then fixed by flooding for five seconds with absolute methyl alcohol containing 0.25 per cent acetic acid. The preparation was then washed in water and stained with a saturated solution of gentian violet in aniline diluted with an equal part of distilled water. The capsules appear as a clear area around the purple bacteria.

Phases 684 A and B, 782 H and Cvl, and 868 F were thought to have small capsules. Although this method is considered by many to be the most satisfactory staining method for the demonstration of capsules on small bacteria, the results obtained here were not consistent. The absence of capsules was not considered reliable.

Variation in Biochemical Activity

All stable phases were inoculated into carbohydrate media prepared as previously described and incubated at 37 C for two weeks. Results are recorded in Table X. Acid was produced consistently from dextrose, mannitol, sorbitol, sucrose, mannose, glycerol, fructose, and galactose. No change was noted in rhamnose, inulin,

TABLE X

FERMENTATION REACTIONS OF STABLE PHASES STUDIED IN THOSE CARBOHYDRATES SHOWING VARIABILITY

Phase	Lactose	Maltose	Raffinose	Dextrin	Starch	Arabinose	Inositol
684 A	A	S	-	-	-	S	A
684 Av1 . .	-	S	-	-	-	S	A
684 Av2 . .	A	A	S	-	-	S	A
684 B	A	A	-	-	-	S	A
684 Bv1 . .	A	A	-	-	-	S	A
710 R1 . . .	-	A	S	-	-	-	-
710 R2 . . .	A	A	S	-	-	-	-
782 C	A	-	S	S	S	S	A
782 Cv1 . .	A	-	S	S	S	S	A
782 H	-	S	S	S	A	-	-
868 E	-	S	S	-	-	-	-
868 C	A	S	S	S	S	S	A
868 F	-	S	S	S	-	-	-
309-all . . .	A	-	S	-	-	-	A
328 S	A	A	S	S	S	-	-

A = acid produced within two days.
S = acid produced in three to seven days.
- = no acid.

salicin, dulcitol, or adonitol. Variable reactions were obtained in lactose, maltose, raffinose, dextrin, starch, arabinose, and inositol. Variability in fermentation did not appear correlated with colonial morphology since identical colony types produced from different cultures did not give identical fermentation reactions.

Variation in Morphology of Cells

Gram stains were made from 24 and 48 hour tryptose agar slants. All phases were gram negative, but much variation in size and shape of cells was noted. Phases 684 B, 782 H, 868 E, and 309 YT were cocco-bacilli typical of pasteurellae. Variation in size of bacilli occurred in 684 Bv1, 782 Cv1, 309 YO', 309 Y, 309 YG, 868 C, and 710 R1. Filaments appeared in 684 Av1, 684 Av2, 782 C, 782 Cv1, 868 F, 868 C, 309 YO, 309 Y, and 309 YG. Involution forms were noted in 684 A, 684 Av1, 684 Av2, 309 Y, 868 C, and 710 R2. 309 YOMO was composed of deep-staining, almost circular forms. Some cells seem to have been lysed in 48 hours leaving a formless residue which stained with safranin. Descriptions of morphology of these phases together with a listing of the gross opacity of growth on an agar slant appears on Table XI. Pictures illustrating the cell types noted will be found in Plates V and VI.

TABLE XI

OPACITY OF GROWTH ON AGAR SLANTS AND DESCRIPTION OF CELLULAR MORPHOLOGY

Phase	Gross Opacity	24-Hour Morphology
684 A	T	Small ovoid bodies, occasional involution form.
684 Av1	T	Pleomorphic, mostly involution forms, few filaments.
684 Av2	T	Mostly bacillary forms, few involution and filamentous forms.
684 B	M	Regular cocco-bacilli.
684 Bv1	M	Regular, cocco-bacilli larger than B.
710 R1	M	Clumped, slender, bacilli of varying length.
710 R2	T	Pleomorphic, mostly involution forms, marked autolysis.
782 C	M	Long, slender bacilli, 50% filaments, few bacilli in chains.
782 Cv1	M	Bacilli varying in length, short filaments present.
782 H	M	Small cocco-bacilli, a few chains.
868 E	M	Short, regular bacilli.
868 C	M	Bacilli, irregular in size, some involution and filamentous forms.
868 F	M	Short bacilli, some long filaments.
309 YT	T	Regular cocco-bacilli.
309 YO	T	Short bacilli, few slender filaments.
309 YO'	T	Fat bacilli, varying in length.
309 Y	T	Chiefly fat bacilli, some involution and filamentous forms.
309 YG	T	Short bacilli, vary in length, some filaments.
309 YOMO	T	Almost circular forms, deeply stained.
328 S	T	Variation in size from frank coccus to definite bacilli, few long filaments.

T = growth transparent.
M = growth more translucent, heavier than T.

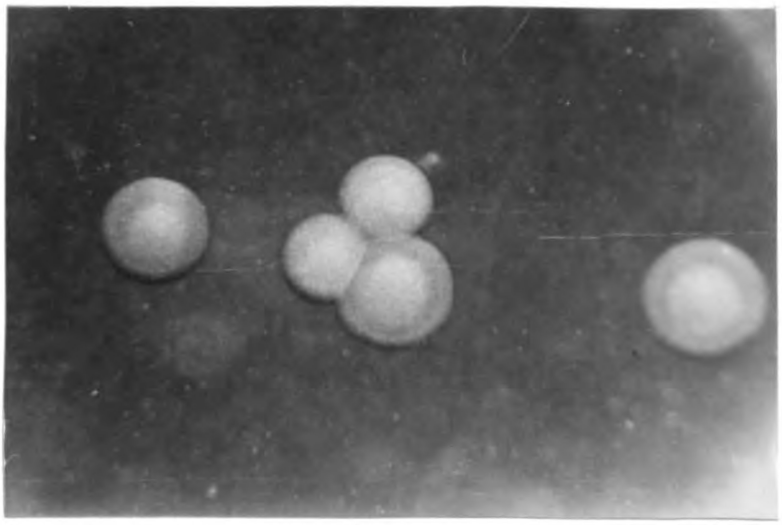
PLATE II

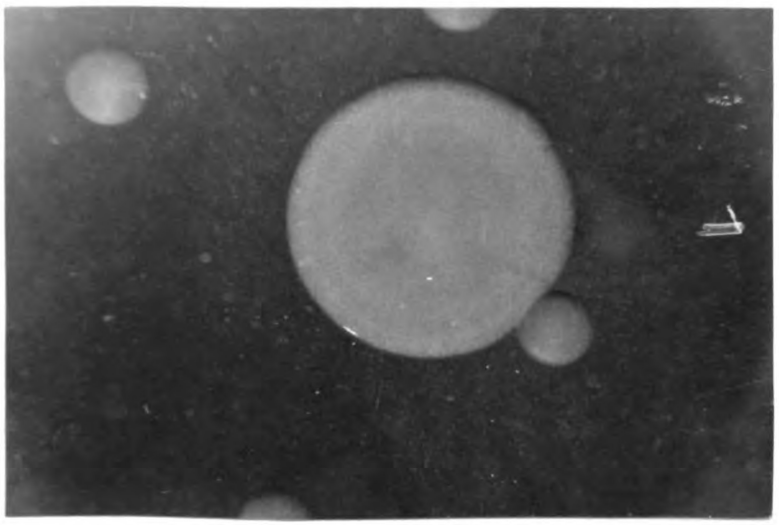
Figure 1. Phases 684 A and 684 Avl.
Note the raised center in the three 684 A colonies and the more opaque and homogeneous appearance of 684 Avl.

Figure 2. Phases 684 A and 684 B.
Note the larger size of 684 B and the homogeneous appearance and reduction in size of 684 A in the presence of B.

Figure 3. Phases 684 B and 782 C.
Note similarity of opacity and contour. Phase C is the colony farthest right.

Magnification: 16X





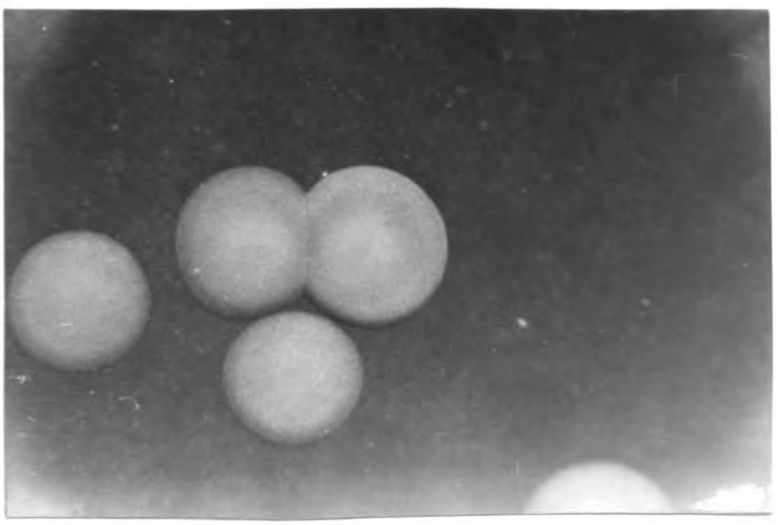


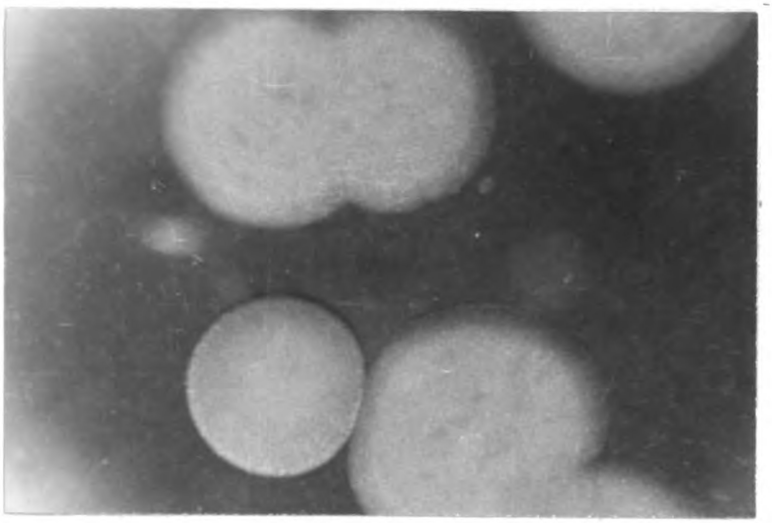
PLATE III

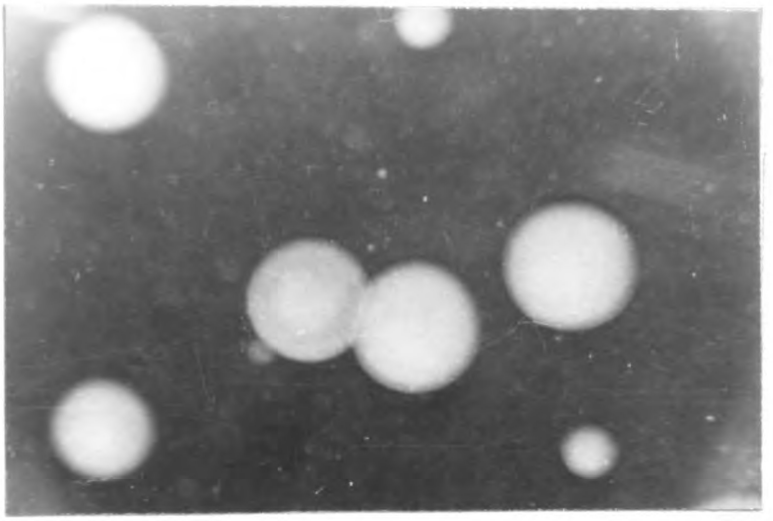
Figure 1. Phases 782 C and 782 H.
Note the thin, narrow margin of Phase 782 H in the lower right corner.

Figure 2. Phases 782 C and 868 E.
Note the greater opacity and very narrow, clear margin of Phase 868 E, the colony to the right and contiguous with 782 C.

Figure 3. Phase 782 C.
Note the pronounced ring margin often seen in well-isolated colonies of this phase and 684 B.

Magnification: 16X





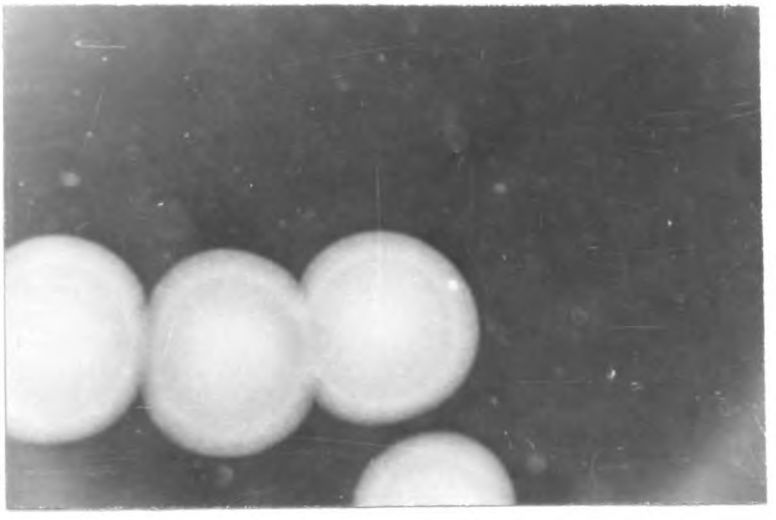


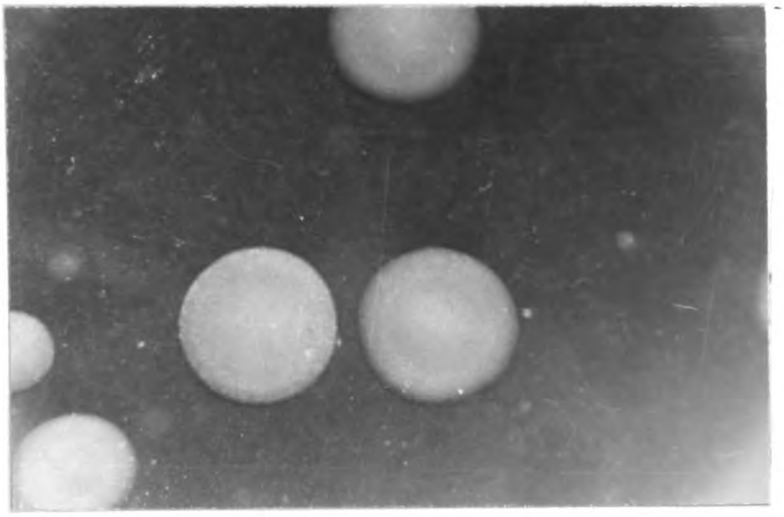
PLATE IV

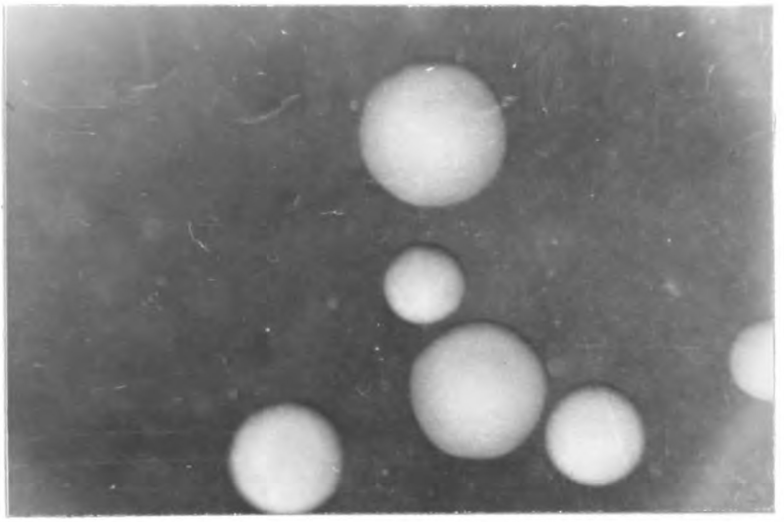
Figure 1. Phases 782 C and 868 F.
Note the softer, looser texture and very slight irregularity of margin in Phase 868 F to the right.

Figure 2. Phases 684 Av1 and 684 Av2.
Note the soft texture and irregular margin of the two (larger) 684 Av2 colonies, and their similarity to 868 F.

Figure 3. Phase 684 BIR. and 684 B.
Note reduction in size and irregular margin typical of Phase BIR.

Magnification: 16 X





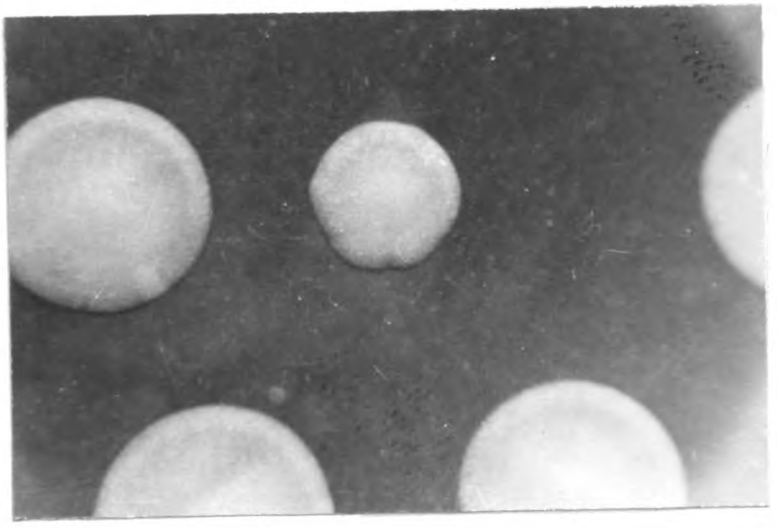


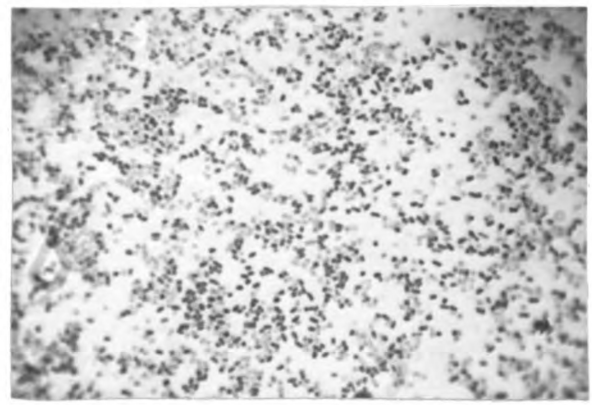
PLATE V

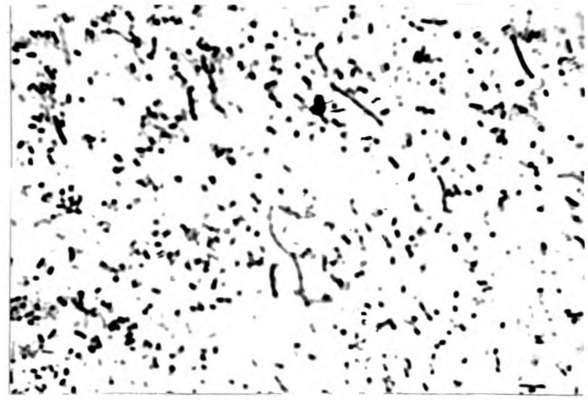
Figure 1. Phase 868 E showing short, regular bacilli typical of pasteurellae.

Figure 2. Phase 868 F showing short bacilli and some slender filaments.

Figure 3. Phase 782 H (48 hours) showing autolysis with disintegration of cellular structure.

Magnification: 1,000X





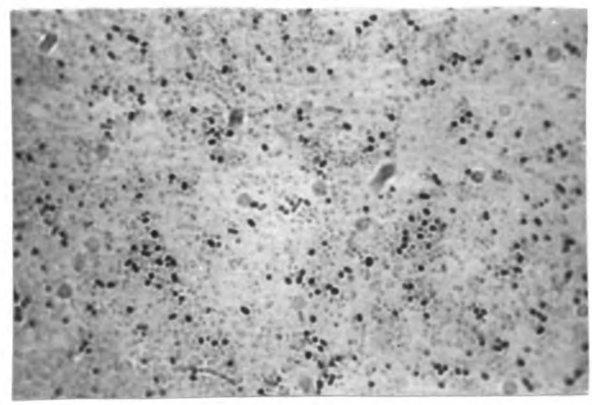


PLATE VI

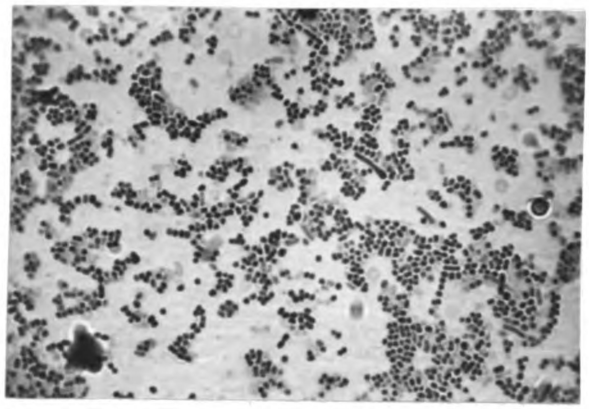
Figure 1. Phase 782 H (24 hours) showing coccoid cells, a few short chains of bacilli, and a clubbed form.

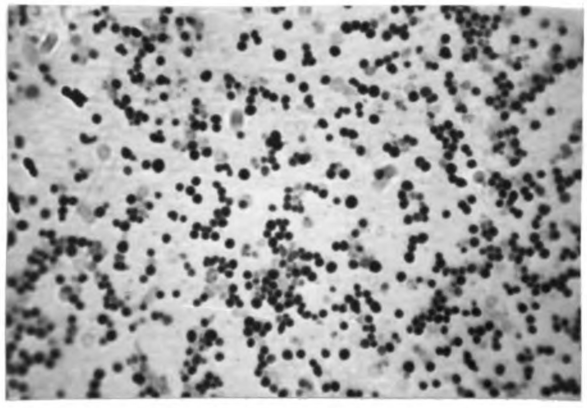
Figure 2. Phase 309 YOMO showing the almost circular and deeply staining cells seen in this phase.

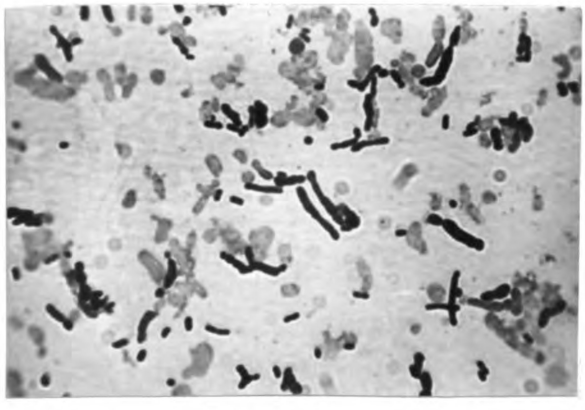
Figure 3. 684 Avl showing involution forms; note the pale-staining, swollen cells as well as the more prominent irregular filaments.

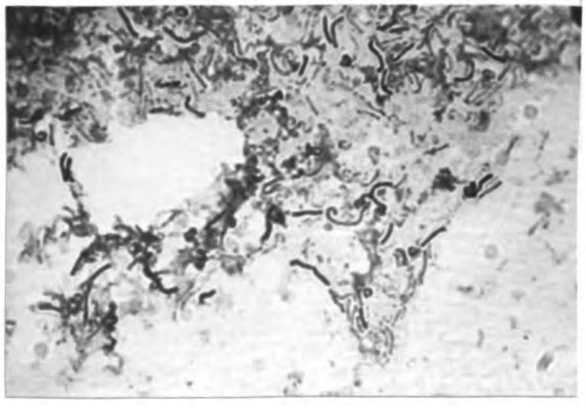
Figure 4. 309 V-2 showing curved filaments.

Magnification: 1,000X









DISCUSSION AND SUMMARY

Studies were made on the serological relationships, fermentation reactions and dissociation patterns of Pasteurella hemolytica strains isolated from chickens. The cultures studied produced acid readily from dextrose, lactose, mannitol, sucrose, galactose, xylose, and mannose. On primary isolation, only a small amount of acid was produced from lactose. A small amount of acid was also produced from sorbitol in 48 hours; this reaction faded and appeared negative after four days. Acid was produced slowly from glycerol, raffinose, and trehalose. A few strains fermented dextrin, starch, and arabinose. Maltose fermentation was variable.

A study of the dissociation phases present in newly isolated cultures revealed a high proportion of intermediate (I) phases. Out of eighteen cultures, one was smooth (S) as evidenced by stability in acriflavin, three were rough (R) as evidenced by spontaneous agglutination in physiological saline, and the remaining fourteen were intermediate. The smooth phase produced a uniformly turbid growth in broth with no pellicle or sediment, remained stable in physiological saline and acriflavin, and was not agglutinated in acid solution. Cellular morphology varied from coccoid forms to definite bacilli.

This variation may have been environmentally induced since the medium used in these studies was not optimum for the growth of this phase. The intermediate phases were very unstable and dissociated into other I phases readily on agar media. They also showed a greater tendency toward sediment and pellicle formation than did the smooth phase. The I phases did not agglutinate in physiological saline, and most of them remained in suspension for 24 hours. Agglutination in acidified saline took place at pH 3 and 4 for most of the phases. Five of the eighteen phases also agglutinated at pH 5. Five other I phases did not agglutinate in acidified saline at any pH. None of the phases stable in saline agglutinated at 80 C. Four of the intermediate phases were stable in 1-500 acriflavine on the slide test but agglutinated in 1-1,000 acriflavine when incubated in tubes at 37 C for six hours.

Variation, independent of colonial changes, occurred in the fermentation of carbohydrates and in cellular morphology. The changes in cellular morphology were marked. Both coccoid and bacillary forms were seen. Filaments were often present, and involution forms, which are typical in old cultures of most pasteurellae, were sometimes present in 24 hours.

Although studies were incomplete due to a laboratory mishap, the presence of more than one serological type of P. hemolytica was

demonstrated by agglutination tests. These types could not be differentiated by correlation with biochemical activity on carbohydrates as were the types demonstrated by Spray and Newsom and Cross in their work with sheep and bovine strains. Inasmuch as the dissociation studies indicated that P. hemolytica strains were seldom smooth when isolated, these serological data are of limited value. The use of I phases for such study may partially account for poor antigenicity noted by most investigators.

Two of the cultures studied exhibited nutritive requirements different from those of the other strains. When 1.0 ml of a suitable saline dilution of broth culture was inoculated onto the two media being used, most of the cultures produced a larger number of colonies on tryptose blood agar base (Difco), containing 1.0 per cent tryptose, 0.3 per cent beef extract, and 0.5 per cent sodium chloride. Strains 309 and 328 evidenced a larger number of viable cells on tryptose agar (Difco) which contains 2.0 per cent tryptose, 0.1 per cent dextrose, and 0.5 per cent sodium chloride. It is not known whether this difference in nutritive requirements is correlated with distinct strains of P. hemolytica or is a characteristic of certain dissociative phases. Strains 309 and 328 were also decidedly more transparent than the other cultures both grossly and microscopically. Whether or not this difference in opacity would always coincide with different

nutritive requirements cannot be stated on the evidence of two cultures. These transparent cultures are isolated from chickens far less frequently than are the more opaque strains.

A truly adequate method for propagating P. hemolytica was not found. The use of artificial media for holding cultures was precarious and time-consuming. Subcultures had to be made every five days to insure survival. The addition of 5.0 per cent blood markedly enhanced growth and facilitated recovery from cultures with few viable cells but did not lengthen the interval between subcultures. The high rate of dissociation as well as difficulty in obtaining suitable growth, difficulty in maintaining viable cultures, and cellular pleomorphism all indicate that the media used were not optimum. Attempts were made to preserve cultures by holding semisolid cultures at -40 C and by lyophilizing in milk, but these methods were not entirely satisfactory either. Recovery on subculture was uncertain, and the presence of whole blood was necessary. A better understanding of the nutritive requirements and more information on enzymes and toxin production should be sought in further studies of P. hemolytica.

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