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A STUDY OF THE ETIOLOGY OF ROUP
IN BIRDS.

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CONCLUSIONS.

From the data submitted, culture "33A" apparently deserves recognition as the etiological factor in roup. The organism has been recognized in smears from all cases of diphtheria and ocular roup which have been examined. It has been grown upon artificial media and the disease again reproduced. Finally, a high degree of protection has been shown against the natural disease after immunization with pure cultures of this organism.

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A Study of the Etiology of Roup in Birds.

JOHN G. JACKLEY.

INTRODUCTION.

Certain facts concerning the etiological relationship of a bacterium of the Pasteurella group to the disease roup in fowls are reported in this bulletin, with secondary emphasis upon a new method for the control of the disease. Studies on the etiology of roup have not been wanting in the past, but the diverse and conflicting results obtained by investigators indicate that the fundamentals have not been reached. In fact, the etiological factor has not been satisfactorily demonstrated, and obviously control methods must remain on an insecure and unscientific basis.

ECONOMIC IMPORTANCE OF ROUP.

A summary of all inquiries pertaining to poultry diseases at the Kansas Experiment Station has disclosed the following approximate percentage relationship of the various diseases in the state: Cholera, 15 percent; roup, 70 percent; white diarrhea, 9 percent; blackhead 1 percent; other troubles (mites, lice, worms, etc.), 5 percent. Losses to the industry from roup vary with different seasons. Occasional outbreaks show a mortality of 50 to 100 percent. In other years only 5 percent of the flock may be infected, with perhaps less than 1 to 2 percent mortality. Recovered cases are believed to be unprofitable; egg production is generally completely suspended for a time; the bird seldom fattens and may die later.

MORBID ANATOMY AND SYMPTOMATOLOGY.

Much controversy has existed in the past regarding the supposed dual form in which this disease manifests itself, viz.: the skin form, contagious epithelioma (pox or sorehead); or the form attacking the mucous membrane of the head, diphtheritic (avian diphtheria, canker, contagious catarrh, ocular

roup or swell-head). Some investigators still believe these two forms to be distinct diseases, although this view is not now generally held.

Contagious epithelioma may be described as a highly contagious disease characterized by hyperplasia of the epithelium of the unfeathered portions of the head, but at times extending to other parts of the body. The wartlike proliferations may spread rapidly until the comb and wattles appear several times their normal thickness. In severe cases the nodules exude a clear fluid and appear moist, somewhat like a cancerous growth. In these cases it is usual to find diphtheria also present. If so, the prognosis is grave. When the nodules are few and scattered, as in mild cases, the bird usually recovers in a week or ten days, apparently without noticeable ill effects.

Diphtheritic roup is characterized by the formation of croupous or diphtheritic membranes upon the mucosæ of the head. When involving only the oral cavity it is commonly called canker or avian diphtheria. When the nasal membranes, together with the sinuses of the head, are involved it is called contagious catarrh, ocular roup, or roup. The latter is at first recognized as an acute rhinitis and conjunctivitis. In a few days the early watery discharge becomes thicker and turbid, the nasal passages become occluded, the eyelids become adherent, and the accumulation of exudate gradually puffs the loose tissue of the orbit. The infraorbital sinus is also often involved. By the seventh to tenth day the crisis of the disease is reached, and the previously doughy swelling becomes firmer and rubbery. The eyelids separate, due to pressure from behind, disclosing a protruding, yellowish-white mass which may attain the size of a hickory nut. The great pressure upon the tissues at this time probably decreases the blood supply, with consequent impairment in the tissue resistance, thus allowing myriads of bacterial invaders opportunity for action—a condition, no doubt, which accounts for the very offensive odor so noticeable about the roup bird.

Persistent efforts to remove the mass by scratching results in more or less severe laceration of the adjoining parts, including at times the eyeball itself. Improvement may or may not begin at this stage. If the case becomes progressively worse, nervous symptoms, probably due to the absorption of poisonous products, develop. The bird refuses to eat, sits

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apart by itself with feathers ruffled and wings drooping, and if disturbed utters a low, distressed cry. Death, preceded by a short comatose period, follows shortly. Recovery, when it occurs, is generally slow, weeks or months usually being necessary. The cornea may remain permanently opaque or the entire eye may disappear. No microscopic lesions in the viscera are evident at autopsy in either pox or diphtheritic roup.

HISTORICAL.

The study of the etiology of roup, according to Hausser, began essentially in the year 1869, when Rivolta investigated an outbreak of the disease and recorded finding "rundliche Kügelchen," which he believed to be protozoa. Subsequently Rivolta and Silvertrini, working together, made further studies and substantiated the original observations of Rivolta. Loeffler (1884) undertook a bacteriological examination of diphtheria in pigeons, and isolated a rod-shaped bacterium belonging to the Pasteurella group, which he named *Bact. diphtheria columbarum*. Loeffler's organism is very similar to one isolated later (1894) by Loir and Duclaux in an outbreak in Tunis, an organism which these authors called *B. diphtheriæ avium*. Moore found an organism belonging to the same group, but could not reproduce the disease. Marshall (1900) studied the disease in Rhode Island. He was unable to reproduce avian diphtheria with the bacillus of human diphtheria, and concluded that protozoa were probably concerned. Guérin (1901) described a cocco-bacillus of the Pasteurella group, but could not reproduce the disease. An important epoch in the study of pox was marked by the year 1902. In this year Marx and Sticker brought proof that the virus of the disease would pass through a porcelain filter. Borrel (1904) confirmed the work of Marx and Sticker. He also described certain cell-inclusion bodies—nonmotile, spherical or biscuit-shaped forms ("pox bodies")—belonging to the protozoa. Harrison and Streit (1904), at the Ontario Agricultural College, report finding the "Roup bacillus," presumably a colon type. Cary (1906) isolated a number of bacteria, *B. coli*, *B. pyocyaneus*, *cocci* and *B. diphtheria columbarum* of Loeffler. Infection experiments using these organisms were not successful. Muller (1906) recognized a rod-shaped bacterium similar to the human diph-

theria organism. Bordet (1907) reported the finding of a bacterium not heretofore described. He gave only a brief morphological description, and ends by saying: "Ce microbe est probablement, avec celui qui provoque la péripneumonie bovine, le plus petit qui ait été cultivé." Carnwath (1908) studied numerous bacteria, among them "der Müllersche Erreger," but neither with it nor any other bacteria could he produce the disease. Lipschütz (1908) verified the presence of the "Borrel'schen Körperchen" in pox lesions. MacFadyean includes the causative agent of pox with the filterable viruses. P. B. Hadley (1909) believed *Coccidia* the cause. Hausser in the same year described a "new bacterium," but Schmidt failed to substantiate any reported causative organisms. Boggero (1910) gave a detailed description of an organism belonging to the Pasteurella group. Schuberg and Schubotz (1910) studied the cell inclusions of Prowazek, "chlamydozoen," but question their relation. Rappin and Vanney (1911) again emphasize Loeffler's bacillus as the cause. Betegh (1912) concludes: "Die erreger der Krankheit sind die von Borrel beschriebenen Körperchen-Strongyloplasma avium, Borrel; und können zu den Protozoen gezählt werden." Schuberg again (1913) states: "Die Erreger sind noch nicht bekannt." Hadley and Beach (1913) failed to substantiate a filterable virus. Beach, Lothe and Halpin (1915), studied an outbreak of the disease; isolated "a secondary invader" belonging to the Pasteurella group. They emphasize its nonspecificity. Phillips during this same year isolated a polar staining organism, which he believed to be the cause. Finally, Brumley and Snook (1916) reiterate that the disease is due primarily to a filterable virus, but admit the importance of certain secondary invading organisms, chief of which is the so-called "*Bacillus diphtheriae colurnbarurn*" of Loeffler. "Neither factor alone will cause the typical disease," according to the investigators.

DEVELOPMENT OF EXPERIMENTAL WORK.

I. ISOLATION OF ORGANISM.

Owing to the large amount of pathological material available, the early efforts in this work were focused almost wholly upon methods and media for isolation. Media of various kinds were prepared to favor poorly growing types of bacteria in the hope of demonstrating the constant presence of some offending species. Standard formulæ for these media were followed in so far as possible. Directions given in Besson's "Practical Bacteriology and Microbiology and Serum Therapy" and "Standard Methods of Water Analyses" were in most cases taken as authority. The following media have been chiefly used in the routine cultural studies:

For aerobic microorganisms:

1. Standard meat infusion agar: neutral to phenolphthalein.
2. Serum agar: 1 cc. sterile horse serum to 9 cc. standard meat infusion agar.

For anaerobic microorganisms:

3. Neutral sugar-free meat broth plus 1 percent glucose, and sterile bits of liver.
4. Neutral liver agar for shake cultures.

Cultural examination of blood and viscera. This work has quite uniformly failed to demonstrate the presence of any form of organism in the blood or viscera of either early or chronic cases of roup. Of twenty-four *post-mortem* examinations, showing different stages of the disease, in only one case did growth develop. Hence it is believed that the causative organism is localized during the course of the disease, and, except in very rare cases, can not be isolated from the blood stream. These exceptions would probably constitute the septicemic form of roup as described by some writers. A considerable portion of the first two years work now serves, unfortunately, only to establish this fact.

*Examination of blood by animal inoculation.*¹ Blood from four cases of ocular roup representing different stages of this

1. Five to 10 cc. of blood can be drawn directly from the heart of a mature fowl without appreciable ill effect. This may be accomplished by means of a large 10 cc. syringe, or preferably by a special suction device.

form of the disease and one advanced case of the diphtheritic form was injected as follows:

Bird 1: 2 cc. breast muscle.

Bird 2: 2 cc. crural space.

Bird 3: 2 cc. peritoneal cavity.

One rabbit and one guinea pig, each 1 cc., axillary space.

At the end of four weeks all animals were well and to all appearance normal. However, the limited number of cases examined make far-reaching conclusions impractical.

Cultural examination of eye lesions. The pus from the eye of the chronic case of roup reveals such a varied flora of organisms that it seemed at first a hopeless task to ascribe significance to any one. It has been a hopeless task in so far as direct isolation has served. Nevertheless, in the early stage of the disease, while only a very slight watery discharge is in evidence, a very small bipolar organism is frequently present in a pure state. A platinum loopful of the fluid may yield a pure culture when smeared upon serum-agar, or even upon a plain agar slant.

Examination of eye lesion by animal inoculations. The caseous, yellowish-white pus surrounding the eye, including parts of the inflamed and thickened nictitating membrane, when ground in a sterile mortar with one to three parts by volume of salt solution, centrifuged at low speed to throw down the heavier particles, gives a resulting milky supernatant fluid which may be used for inoculation purposes. (This suspension is referred to subsequently as natural virus.) One-fourth to one-half cc. of the suspension introduced into the axillary space of a rabbit is fatal in twenty-four to forty-eight hours, producing a typical septicemic infection. From the viscera and blood, pure cultures of the polar staining organism previously described may be obtained. Hens, pigeons and guinea pigs when heavily inoculated with this fluid occasionally die of septicemia, and the organism may be recovered from the heart blood. Guinea pigs and white rats, especially the latter, are very refractory and do not lend themselves readily to use for isolation purposes.

Filterability of the virus. Berkefeld filtrates (filter "N") of three virus suspensions failed to affect any of a series of hens, rabbits, doves and guinea pigs, despite the fact that each suspension before filtering was fatal to rabbits upon subcutaneous injection.

II. CULTIVATION OF ORGANISMS.

Cultivation of the organism has already been referred to above. Growth is slight at first on plain agar, but better on serum-agar. (See study of organism for particulars of growth.)

III. REPRODUCTION OF ORIGINAL DISEASE.

One of the chief obstacles encountered early in this work lay in the reproduction of the typical disease. Bordet (1907) referred to this difficulty when he said: "Beaucoup de microbes ont été décrits comme provoquant la diphthérie des poules ou des pigeons. Mais on doit remarquer quaucun des micro-organismes signalés ne reproduit bien exactement la maladie naturelle, etc." Until very recently, satisfactory reproduction of the disease, even with natural virus, was not successful, hence no *modus operandi* existed for reproducing it with pure cultures.

Natural virus, when introduced according to the conventional methods (subcutaneous, intraperitoneal, intramuscular, intrapleural or feeding), has not generally proved pathognomonic for hens; when death occurs it is without the development of local lesions in the eye or mouth. Scarifying the mucous membranes of the eye or mouth, using a hot, heavy platinum wire loop, with subsequent rubbing in of the virus, gave inconstant results. Slitting the eyelid with a scalpel and later injecting virus into the wound was successful in reproducing the typical eye tissue changes, but was cumbersome. A simpler and easier method is a modification of the "Bordet infection technique" (Bordet, 1912): A piece of common wrapping twine threaded to a large curved suturing needle (such as is used by the veterinary profession) is autoclaved. By means of a sterile mortar and pestle the string is saturated with the "virus" suspension. The needle is then used to pierce the eyelid of the bird, catching one-eighth to one-fourth inch from the edge. The free ends of the string are tied so that a small loop is formed, thereby allowing opportunity for tissue expansion.

Following this infection method a rapid succession of changes takes place, beginning with the clear, watery eye discharge of the natural infection, and duplicating in turn all of the various subsequent stages as these occur in the natural course of the disease.

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The results of the Bordet infection technique were so uniformly successful that an experiment was outlined to test this infection method, using pure cultures of organisms instead of the natural virus. Simple as this would appear, it was nevertheless one of the most vexing problems encountered in the work. From routine plating and other isolation methods several different organisms had been obtained. A brief cultural and morphological study of these is shown below:

1. Culture 33A: small, nonmotile, diplococcus-like rod; no spores; Gram negative; no gas in carbohydrates.
2. Culture 33B: motile rod; Gram negative; no spores; acid and gas in glucose and lactose broth (probably some variety of *B. coli*).
3. Culture 33C: motile rod; Gram negative; producing a soluble green pigment (probably *B. pycocyaneus*).

Each of these or similar organisms has at different times been critically studied and ascribed a rôle in the etiology of the disease.

These three cultures were substituted for the natural virus in the "Bordet infection method," using twenty-four-hour-old broth cultures. The results are shown in Table I. Observations were made at the end of one, two, four and seven days, two weeks and three weeks. If at any time, from one day on, a roupy eye developed, it is marked "+"; no change, "-"; and indefinite reaction, *i. e.*, swelling and reddening of the eyelids, with some discharge, but not a fully characteristic roupy change, "±." A star (*) in connection with a symbol marks the death of the bird. Vertical columns represent the number of the bird infected; horizontal columns, the innoculum used.

TABLE I.
 DATE, April 1, 1916.

	1	2	3	4	5	6
Culture 33A.....	*+	*-	+	=	+	+
Culture 33B.....	=	-	=	+	-	-
Culture 33C.....	-	+	-	*±	-	-

*Died.

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Several questions were suggested by the results of this table: (1) Is roup caused by *any one of these organisms?* Each one has produced at least some roup symptoms. It is noteworthy that culture 33A gave a much higher percentage of positive infections. However, both 33B and 33C gave one positive infection each. (2) Is roup, as has been suggested, not a specific infection? Are the facts as given in Table I a corroboration of this? In order to give this point a more thorough test, a second experiment was planned, conducted as before, but using a series of laboratory pure cultures, including, in addition, certain controls which had been suggested. All cultures were subjected to one week's rejuvenation process before use (daily transfers from broth to agar slant and back to broth, etc.). The results are shown in Table II.

TABLE II.

	1	2	3	4	5	6	7	8	9	10	11	12
1. <i>B. coli</i>	*+	-	-	-	*±	-	-	±	-	-	-	-
2. <i>B. prodigiosus</i>	-	-	*+	-	-	-	±	-	-	-	-	-
3. <i>B. pyocyaneus</i>	-	-	*-	-	*±	-	-	+	-	-	-	-
4. <i>B. paratyphosus A</i>	-	-	-	+	-	-	-	+	-	-	-	-
5. <i>B. paratyphosus B</i>	-	-	±	-	*+	-	-	-	-	-	-	-
6. <i>B. avisepticus</i>	-	-	*+	-	+	*-	±	-	+	-	-	-
7. <i>B. bovisepiticus</i>	-	*+	-	-	*+	*-	-	*±	-	*-	-	-
8. <i>B. pullorum</i>	-	-	-	*+	-	-	-	±	-	-	-	-
9. Culture 33A	+	+	*+	-	+	*±	-	+	+	*-	*+	+
10. Sterile string only	±	-	-	-	±	+	-	-	*-	±	-	-
11. Sterile broth	-	±	-	+	-	-	-	*±	-	+	-	-
12. Sterile salt solution	-	-	-	-	+	-	-	-	±	-	±	-

*Died.

Strangely enough, the control birds here show a number of indefinitely positive reactions and one or two typical reactions. Similarly, a number of these old laboratory cultures gave positive infections in addition to a number of indefinite reactions. These results were puzzling. Must we assume that almost any organism, if it gains entrance to the tissue of the bird, may, when the vitality is lowered, produce roup symptoms? Birds as a class are very resistant to bacterial invasion. Then, too, this does not explain the positive cases occurring in the controls, nor why culture 33A is seemingly so much more active than the other cultures. After considerable thought the idea was suggested that perhaps the causative agent might be present, *e. g.*, in the dust, litter and fecal matter of the cage. It had often been observed that immediately following the

threading operation there occurred a period of violent scratching by the bird, during which time much dirt probably gained entrance to the artificial wound. Could it be possible that this dust and dirt harbored the organism and the disturbing and contradictory results be explained thereby? A third experiment seems to answer this point.

Twelve birds were infected by the eye-string method from several cultures. The legs of each bird were securely bound, after infection, with cloth bands to prevent scratching; otherwise methods were followed identical to those in the previous experiments. Table III gives the results:

TABLE III.
DATE, October 2, 1916.

	1	2	3	4	5	6	7	8	9	10	11	12
33A.....	±	+	*+	*+	±	+	*-	+	*+	-	*±	+
<i>B. coli</i> (33B).....	-	-	-	-	-	-	±	-	-	-	-	-
<i>B. pyocyaneus</i> (33C).....	-	-	-	-	-	±	-	-	-	-	-	-
<i>B. avisepticus</i> (AM).....	-	-	*-	-	-	*-	-	-	±	-	-	-
Sterile string.....	-	-	-	-	-	-	±	-	-	-	-	-

*Died.

Culture 33A again shows a high percentage of positive infections, and the other cultures and the controls show no positive infection.

The interpretation of this tabulation, together with those just preceding it, appears to be: (1) organism 33A is the causative factor. (2) That organism must be present in the dust and litter, and is carried on the feet of the bird to the eye, the string serving as a slight irrigation and focus for initial growth. When the feet are tied this extraneous infection is rendered impossible. (3) The organism is presumably powerless to act upon the intact mucous membrane of the eye.

IV. PROTECTIVE INOCULATIONS.

Bacterins, vaccine, antisera and the antitoxin of Roux and Behring for human diphtheria have all been repeatedly tried in avian diphtheria, roup and pox, both for their curative and prophylactic value. The following study of protective inoculations is not primarily to determine methods for control, but to determine the specificity of the organism through immunization with pure cultures and subsequent demonstrations of

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protection against the natural disease. The infectiousness of roup, however, is so variable a factor that but little reliance can be given it. On numerous occasions advanced cases of roup have been placed in the same cage with normal birds under the most insanitary and unhygienic conditions and yet no epizootic or even sporadic cases occurred.

Propagation of a fixed virus as a substitute for the natural disease. A method has already been described for artificially reproducing the disease by means of natural virus; but in order to insure the reliability of that method in the following experiments, the virus selected was successively passed through six birds, as follows: A string was saturated with the eye discharge at the height of the acute stage (just before the dry, cheesy pus formation began), and immediately a second bird was infected, and in the same manner a third from this second, and so on. Thus, two strains of virus were developed. One was originally derived from a case of avian diphtheria, the other from ocular roup. These will be referred to as "fixed virus" 200 and 207, respectively.

In the following protocol a series of experiments were undertaken to demonstrate if possible (1) whether a culture of 33A, as an immunizing product, possessed protective power; and (2) to verify, if possible, statements regarding the value of certain bacterins and vaccines as reported by other investigators. Separate groups of birds were immunized with four to five doses of the several products to be described, and after a lapse of seven to ten days were infected by the eye-string method with "fixed virus" 200 and 207. All injections were made intraperitoneally except where otherwise noted. Results are indicated by the same symbols as in Tables I, II, and III. Birds that died before the end of forty-eight hours usually showed no well-marked lesions of roup. These cases were believed to be the septicemic form of the disease, and are indicated as positive roup.

PRODUCT NO. 1.²—Salt-solution suspension of "ground-up" diphtheritic membranes from eye and throat, attenuated by heating to 55° C. for sixty minutes. Five-tenths percent carbolic acid was added as a preservative. Suspensions from ten cases were "pooled" before use. The resulting fluid appeared milky.

2. According to directions as recommended by Hadley and Beach (1913) for controlling chicken pox.

TABLE IV.

BIRD LEG BAND No.	Vaccination product No. 1.					Virus infection. Dec. 11, 1916.	Post-infection observations.						Remarks.
	11- 9.	11- 14.	11- 20.	11- 27.	12- 4.		1d.	2d.	3d.	4d.	7d.	2 wks.	
	cc.	cc.	cc.	cc.	cc.								
375.....	2	3	3	5	10	207	+						+
385.....	2	3	3	5	10	200	±	*+					++
380.....	2	3	3	5	10	200	*-						++
369.....	2	3	3	5	10	207	*±						++
389.....	2	3	3	5	10	207	+		*+				+
379.....	2	3	3	5	10								Died, 12-5-'16.
388.....	2	3	3	5	10	207	±		*+				+
387.....	2	3	3	5	10	207	*±						++
375.....	2	3	3	5	10	207	+	*+					+
5404.....	Control.....					207	+			+			+
5447.....	Control.....					207	+	*+					++
8004.....	Control.....					200		*±					++
5414.....	Control.....					200	+		*+				+

*Died. †Septicemic roup.

PRODUCT No. 2.³—Cultures were made from the base of the diphtheritic membranes of the mouth on neutral agar, grown for twenty-four to thirty-six hours at 37.5° C., washed off in sterile salt solution, attenuated by heating to 55° C. for one hour. The suspension used was very heavy, appearing almost as opaque as whole milk.

TABLE V.

BIRD LEG BAND No.	Vaccination "p" product No. 2.					Virus infection. Dec. 11, 1916.	Post-infection observations.						Remarks.
	11- 9.	11- 14.	11- 20.	11- 27.	12- 4.		1d.	2d.	3d.	4d.	7d.	2 wks.	
	cc.	cc.	cc.	cc.	cc.								
391.....	0.2	0.5	1	1	1	200	+			*+			+
384.....	0.2	0.5	1	1	1	207	-	*-					++
392.....	0.2	0.5	1	1	1	200	*+						+
393.....	0.2	0.5	1	1	1	207	±		+	+	+		+
394.....	0.2	0.5	1	1	1	200	*±						++
395.....	0.2	0.5	1	1	1	207	±		+	+	+		+

Controls: See Table IV.

*Died. †Septicemic roup.

3. Recommended by Brumley and Snook (1916) for the treatment and control of roup.

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PRODUCT No. 3⁴ was a four-day-old broth culture, *Bact. avisepticus* (American Museum),⁵ killed without heat by the addition of five-tenths of one percent carbolic acid. The first immunizing dose consisted of one-half cc. of a living (?) broth culture (carbolyzed one hour before use). Succeeding doses were proved to be sterile by transferring one-half cc. to bouillon for evidence of growth, also by injecting a rabbit with one cc. One cc. of this culture, when used previously as a twenty-four-hour growth in broth, was fatal to hen No. 748, as a subcutaneous infection, in eight days.

TABLE VI.

BIRD LEG BAND NO.	Vaccination product No. 3.				Virus infection. Dec. 11, 1916.	Post-infection observations.					Remarks.
	11- 14.	11- 21.	11- 27.	12- 4.		1d.	2d.	4d.	7d.	2 wks.	
	cc.	cc.	cc.	cc.							
726.....	0.5	1	3	5	200	+	+	+	+
3271.....	0.5	1	3	5	207	+	+	+	+
3267.....	0.5	1	3	5	200	+	*+	+	+
728.....	0.5	1	3	5	207	=	+	+	+
727.....	0.5	1	3	5	200	=	*+	+

Controls: See Table IV. *Died.

PRODUCT No. 4 was an agar-slant growth, culture 33A suspended in carbol-salt solution heated to 60° C. for fifteen minutes. On November 20 a new suspension was prepared of double strength. On December 4 a living twenty-four-hour agar-slant growth, salt suspension, was used in the amount shown.

PRODUCT No. 5 was a four-day-old broth culture of organism 33A, killed without heat by addition of .5 percent carbolic acid; sterility test as for product III. The first six birds received intraperitoneal injections; last six received subcutaneous (breast).

General summary and conclusions from protective inoculation experiments. Product Nos. 1, 2, 3 and 4 show no protection. Product No. 5 has shown complete protection, which is

4. Culture 33 has so many common characters with *Bact. avisepticus* that it was thought that a possible means of identification or differentiation might be made by reciprocal inoculations.

5. Cultural and biochemic reactions of this organism do not agree fully with that generally accepted for *Bact. avisepticus*. See comparative study of this organism later.

TABLE VII.

BIRD LEG BAND No.	Vaccination product No. 4.					Virus infection. Dec. 11, 1916.	Post-infection observations.						Remarks.
	11- 8.	11- 13.	11- 20.	11- 27.	12- 4.		1d.	2d.	3d.	4d.	7d.	2 wks.	
372.....	cc. 1	cc. 2	cc. 2	cc. 3	cc. 0.5	200	*+						+
381.....	1	2	2	2	3	0.5							Died 11-9-'16.
371.....	1	2	2	2	3	0.5	200	=		+	+	+	+
374.....	1	2	2	2	3	0.5	207	+		+	+	+	+
368.....	1	2	2	2	3	0.5	207	+		+	+	+	+
376.....	1	2	2	2	3	0.5	200	=		+	+	+	+
373.....	1	2	2	2	3	0.5	200	=		+	+	+	+
386.....	1	2											Died 11-14-'16.
382.....	1	2	2	2	3	0.5	200	+		+	+	+	+
370.....	1	2	2	2	3	0.5	200	+		+	+	+	+
300.....	1	2											Died 11-14-'16.
388.....	1	2	2	2	3	0.5	207	+		+	+	+	+

Controls: See Table IV. *Died.

TABLE VIII.

BIRD LEG BAND NO.	Vaccination product No. 5.					Virus infection. Dec. 11, 1916.	Post-infection observations.					Remarks.	
	11- 15.	11- 21.	11- 27.	12- 4.	1d.		2d.	4d.	7d.	2 wks.			
3756.....	cc. 2	cc. 2	cc. 5	cc. 10		200		-	-	-	-	-	-
3987.....	2	2	2	5	10	200		-	-	-	-	-	-
3276.....	2	2	2	5	10	200		=	-	-	-	-	-†
3954.....	2	2	2	5	10	207		=	-	-	-	-	-†
3259.....	2	2	2	5	10	200		-	-	-	-	-	-
3268.....	2	2	2	5	10	207		-	-	-	-	-	-
3754.....	1	1	1	2	3	207		-	-	*-			†††
3963.....	1	1	1	2	3	200		-	-	-	-	-	-
3966.....	1	1	1	2	3	207		-	-	-	-	-	-
2366.....	1	1	1	2	3	207		+	+	*+			+
3251.....	1	1	1	2	3	207		-	*				+
3253.....	1	1	1	2	3	200		*	-				+

Controls: See Table IV. *Died.

†Nos. 3276 and 3954 each showed a slight water discharge at forty-eight hours, but had recovered completely by four days.

††Nos. 3754, 3965 and 3996 had been used two months previously in an unfinished experiment, at which time they had each received two injections intraperitoneally of forty-eight-hour-old broth cultures of culture 33A. No. 3754 died suddenly December 13, 1916, with no symptoms of roup. Through an oversight no autopsy was performed, but it is believed that the bird expired from some cause other than from the test infection. In lieu of positive proof, however, it is marked "+."

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interpreted as added proof of the etiological relationship of culture 33A.

It is noteworthy that the subcutaneous injections of product No. 5, if not devoid entirely of protection, were very much less effective than the intraperitoneal. Whether this low degree of protection is entirely due to the method of introduction can not be definitely determined, since the injections were each approximately one-half or less that used in the intraperitoneal method. Absorption is probably considerably slower from a subcutaneous injection, and there may be a slightly less antibody formation.

V. DESCRIPTION OF ORGANISM—CULTURE 33A.

It is at once evident from the historical resumé already given that members of the Pasteurella group of bacteria have been most often suspected as the causative factor of roup. The descriptions are often very meager, but usually coincide more or less closely with the organism here described, which in turn is similar in its morphology, cultural and biochemic reactions to *B. avisepticus*, differing only with respect to degree of pathogenesis.

Considering the unsettled state of knowledge regarding the various members making up this group, it has seemed advisable to await a more comprehensive and comparative study before arriving at any definite conclusion concerning the determination of species. Buchanan, in the last edition (1916) of his "Veterinary Bacteriology," says of the Pasteurella group: "Since it has thus far not proved possible to differentiate the organisms associated with the various diseases by means of their morphologic or biologic characters, it is necessary to adopt tentatively a pathologic classification."

For the present a tabulation is given (Table IX) of the principal cultural and biochemic characters of culture 33A, which is typical for eighteen other pure cultures of this organism studied. *Bact. avisepticus* (American Museum strain, used in the protective inoculation experiments) is also tabulated. A recent exhaustive study of five members of the Pasteurella group by Besemer (1916) is shown for comparative purposes, as is similar study of ten strains of *Bact. avisepticus*, by Mack and Records (1916). Finally, several organisms described by previous investigators as the cause of roup are given.

TABLE IX.

	Culture 33A (18 strains)	<i>B. anthracis</i> Mack and Records (10 strains)	Pastourelia group. Beesmer (5 members)	<i>B. anthracis</i> (American Museum strain)	<i>B. garranus</i> Harrison and Streik	<i>B. thibetica colum- barum</i> Loeffler (Flügge)	<i>B. thibetica artem.</i> Loir and Duclaux (Flügge)	Brunley and Snock
MORPHOLOGY:								
Short rod	++	++	..	++	+	++	++	++
Bipolar staining	++	++	..	++	+	++	++	++
Gram stain	++	++	..	++	+	++	++	++
Motility	+	+	..	+	+	+	+	+
GROWTH:								
Neutral extract agar	+	+	..	+	+
Neutral meat agar	+	+	..	+	+
+ 1. meat agar	+	+	..	+	+
+ 2. meat agar	+	+	..	+	+
+ 3. meat agar	+	+	..	+	+
Endo agar	+	+	..	+	+
10 percent serum agar	+	+	..	+	+
Plain potato	+	+	..	+	+	+	#	..
Glycerine potato	+	+	..	+
Extract broth	+	+	..	+
Peptone water	+	+	..	+
Indol peptone H2O	+	?	..	+	+
Indol sugar-free broth	+	+	..	+	+
Milk—no change	+	+	..	alk.	+
Milk—coagulated	+	+	..	+	+	+
Nitrates reduced	+	+	..	+	+	+
Gelatin liquefied	+	+	..	+	+	+
Pellicle on Br	#	#	..	+	+	+
FERMENTATION OF HYDRATES—								
<i>Acid only:</i>								
1 percent dextrose	+	+	+	+	gas.	+
1 percent lactose	+	+	#	+	+
1 percent maltose	+	+	+	+	+
1 percent saccharose	+	+	+	+	+
1 percent levulose	+	+	+	+	+
1 percent galactose	+	+	+	+	+
1 percent mannite	+	+	+	+	+
1 percent dextrin	+	+	+	+	+
1 percent salicin	+	+	+	+	+
1 percent inulin	+	+	+	+	+
1 percent glycerine	+	+	+	+	+
1 percent dulcete	+	+	#	+	+
1 percent soluble starch	+	+	..	+	+
PATHOGENESIS:								
Subcutaneous—hens	#	#	..	#	..	#	+	#
Subcutaneous—doves	#	+	..	#	..	#	+	..
Subcutaneous—rabbits	+	+	..	#	..	+	+	..
Subcutaneous—guinea pigs	+	#	+	+	..
Subcutaneous—white rats	+	+	+	+	..

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Polar staining has been determined in each case by making smears directly from the heart blood of a rabbit, at time of isolation of the organism, fixing in absolute methyl alcohol and staining in dilute carbol-fuchsin or Giemsa stain.

Indol was tested for by the Ehrlich and the Salkowski-Kitasato methods. The former gave uniformly strong reactions, while the latter shows often only a trace or no indol. In comparing Dunham's peptone water and sugar-free meat infusion broth for indol it was found that the organisms refused to grow in peptone water; consequently all tests recorded for culture 33A were made on sugar-free meat infusion broth cultures.*

Fermentation of carbohydrates. (The medium used was sugar-free meat infusion broth plus 1 percent of the respective carbohydrates.) Acid was determined by titrating, using N/100 NaOH and 1 cc. of media with 10 cc. neutral distilled water for dilution.

Pathogenesis. The pathogenesis has not been fully worked out in the case of culture 33A. It appears to be but moderately or slightly pathogenic to hens and doves. White rats and guinea pigs show considerable resistance. Rabbits are very susceptible. Other animals have not been tested.

Production of toxic substances. Results to date have not been entirely satisfactory. However, in one series, of guinea pigs, inoculated subcutaneously with 1 cc. amounts of carbolyzed four-day-old broth culture (product No. 5), a gradual emaciation and cachexia developed, ending in death after about four weeks. This work is to be repeated in the near future.

Serum reactions. Specific agglutinins for culture 33A could not be demonstrated in the blood serum of three natural cases of roup (pooled serum). Birds artificially immunized to culture 33A gave agglutination in dilutions of $\frac{1}{8000}$. Suspensions of two strains of *B. avisepticus* (Mack and Records) were agglutinated in the same dilutions. Bactericidal properties in the blood serum were not shown to be present, against culture 33A. The complement fixation test when tried was not satisfactory.

6. Preparation of sugar-free broth: The meat infusion obtained by extracting 500 grams lean ground meat, in the ice box over night, with 1000 cc. of distilled water, is heated to boiling and filtered to remove the coagulum. When the filtrate is cool it is inoculated with a broth culture of *B. saccharolyte* and incubated over night. On the following morning steam fifteen minutes, add peptone and salt in the usual proportion, steam again, and adjust reaction to plus .2. The finished product should produce no gas when inoculated with the organism mentioned and should give no test for indol.

BIBLIOGRAPHY.

- BEACH, B. A., LOTHE, H., and HALPIN, J. G. 1915. An outbreak of roup and chicken pox in which the high mortality was apparently caused by a secondary invader. In *Jour. Infect. Diseases*, vol. 17, No. 3, pp. 554-558.
- BESSON, A. 1913. *Practical Bacteriology Microbiology and Serum Therapy*. Longmans, Green & Co.
- BÉTEGH, L. VON. 1912. Ueber die Beziehungen zwischen Geflügel Diphtherie and Geflügelpocken. In *Centralbl. f. Bakt. Abt. I, Orig.* 67, S. 43-50.
- BESEMER, A. M. 1916. A study of five members (or so-called species) of the septicemia hemorrhagica (*Pasteurella*) group of organisms with special reference to their action on the various carbohydrates. In *Jour. of Bact.* V, II, No. 2, pp. 177-184.
- BOGGERO, A. 1910. Osservazioni sulla differite aviaria. In *Centralbl. f. Bakt. Abt. I*, 50, S. 243.
- BORDET, M. 1907. Recherches sur la diphtérie aviare. In *Soc. Roy. des Sciences Med. et Nat. Brux. Bull. des Sciences* 65.
- BORDET, J., et FALLY, V. 1910. Le microbe de la diphtérie des poules. In *Ann. de l'Inst. Pasteur T.* 24, pp. 563-68.
- BORDET, J. 1912. La diphtérie des pigeons. In *Centralbl. f. Bakt. Abt. I, Orig., Bd.* 67, S. 41.
- BRUMLEY, O. V., and SNOOK, J. H. 1916. Epitheliosis infectiosa avium; contagious epithelioma; diphtheria; roup; canker. In *Vet. Alumni Quart.* (Ohio State University), Vol. 3, No. 3.
- CARNWATH, T. 1908. Zur Aetiologie der Huhnerdiphtherie und Geflügelpocken. In *Arb. d. Kaiserl. Gesundheitsamte Bd.* 27, Heft.
- CARY, C. A. 1906. Chicken pox or sorehead in poultry. In *Alabama College Sta. Bull.* 136.
- GUÉRIN, C. 1901-1903. Sur la non-identite de la diphtérie humain et de la diphtérie aviare. In *Recueil de Med. Vet.* (1903) p. 20, and in *Past. Inst.* T15 (1901) pp. 941. Cited by Besson.
- HADLEY, F. B., and BEACH, B. A. 1913. Controlling chicken pox, sore head or contagious epithelioma by vaccination. In *Amer. Vet. Rev.* 44, No. 3, pp. 330-339.
- HADLEY, P. B. 1909. Roup in fowls. In *Centralbl. f. Bakt. Abt. I, Orig. Bd.* 50, S. 348.
- HARING, C. M., and KOFOID, C. A. 1912. Observations concerning the pathology of roup and chicken pox. In *Centralbl. f. Bakt. Ref.* 53, S. 241.
- HARRISON, F. C., and STREIT, H. 1902-1904. Roup. In *Ontario Agr. Col. and Exper. Farm Bul. No.* 125, *Ibid. Bul. No.* 132.
- HAUSSER, A. 1911. Bacteriologische Untersuchungen über Geflügel-diphtherie. In *Centralbl. f. Bakt. Abt. I, Orig. Bd.* 48, Heft 5, S. 535.

A Study of the Etiology of Roup in Birds. 23

- LIPSCHUTZ, B. 1908. Untersuchungen über Epithelioma Contagiosum der Vögel. In *Centralbl. f. Bakt.*; Abt. I, Orig, 46, S. 609.
- LOEFFLER. 1884. Untersuchungen über die Bedeutung der Mikroorganismen für die Entstehung der Diphtherie beim Menschen bei der Traube und beim Kalb. In *Mitt. aus dem Kais. Ges. Amt*, Bd. II, S. 421.
- LOIR ET DUCLAUX, A. P. 1894. Contribution a l'etude de la diphtérie aviaire en Tunisie. In *Ann. de l'Inst. Pasteur*, T8, 599.
- MARX, E., und STICKER, A. 1902. Untersuchungen über das Epithelioma contagiosum des Geflügels. In *D'tsche Mdz, Wochenschr.* S893.
- MACK, W. B., and RECORDS, E. 1915. The control of contagious epithelioma in chickens by vaccination. In *Bull.* 82, Nevada Exper. Sta.
- MACK, W. B., and RECORDS, E. 1916. The use of bacterins in the control of fowl cholera. In *Bull.* 85, Nevada Exper. Sta.
- MARSHALL, H. W. 1900. Notes on roup. In *Rhode Island Rpt.* pp. 233-244.
- MANTEUFEL. 1910. Beiträge zur Kenntnis der Immunitätserscheinungen bei den sogenannten Geflügelpocken. In *Arb. a. d. Kais. Gesundheitsamte* Bd. 33, H3, S305. (Abstract *Centralbl. f. Bakt. Abt. I*, Ref. Bd. 49-548.)
- McFADYEAN, J. 1908. The ultraviolet viruses. In *Jour. Compar. Path. and Ther.*, 21, Nos. I, pp. 58-68; II, pp. 168-175; III, pp. 232-242.
- MOORE, V. A. 1885. A preliminary investigation of diphtheria in fowls. In *Bull. No. 8*, U. S., B. A. I.
- MULLER, R. 1906. Zur Aetiologie der Geflügel diphtherie. In *Centralbl. f. Bakt. Abt. I*, Orig. 41, pp. 432-515, 621.
- PHILLIPS, A. G. 1915. A preliminary investigation with roup. In *Jour. of the Amer. Inst. and Invest. in Poultry Husbandry*, vol. I, No. 4, pp. 28-31.
- RAPPIN and VARNEY, A. 1911. Sur l'identité des diphtérie aviaires et humaines. In *Compt. Rend. Soc. Biol.* (Paris) 70, No. 5, pp. 162-168.
- RATZ, S. 1913. Investigations of the filterable virus of avian diphtheria and contagious epithelioma of birds. In *Monatsch. Prakt. Tierheilk.*, 25, Nos. 1-2, pp. 41-46. (Abstract *Exper. Sta. Record*, 1915.)
- SCHMIDT, G. 1909. Untersuchungen über die Beziehungen zwischen Geflügeldiphtherie und Epithelioma Contagiosum. In *Centralbl. f. Bakt. Abt. I*, Orig. 52, Heft. 2, S200.
- SIGWART. 1910. Experimentelle Beiträge zur Frage der Identität von Geflügeldiphtherie und Geflügelpocken. In *Abt. I*, Orig. 56, S428.
- SHUBERG und SCHUBOTZ. 1910. Zur Frage der Geflügelpocken. In *Centralbl. f. Bakt. Abt. I*, Bd. 47, S108. Behaft.
- SCHUBERG. 1913. Weitere Beiträge zur Kenntnis der Geflügelpocken. In *Centralbl. f. Bakt. Ref.* 59, p. 222.
- SWEET, C. H. 1913. A study of epithelioma contagiosum of the common fowl. In *Uni. Cal. Pubs. Zoöl.*, vol. II, No. 3, pp. 29-51.