

MASSACHUSETTS
AGRICULTURAL EXPERIMENT

BULLETIN No. 295

MAY, 1933

The Immunology of
Infectious Laryngotracheitis

By Charles S. Gibbs

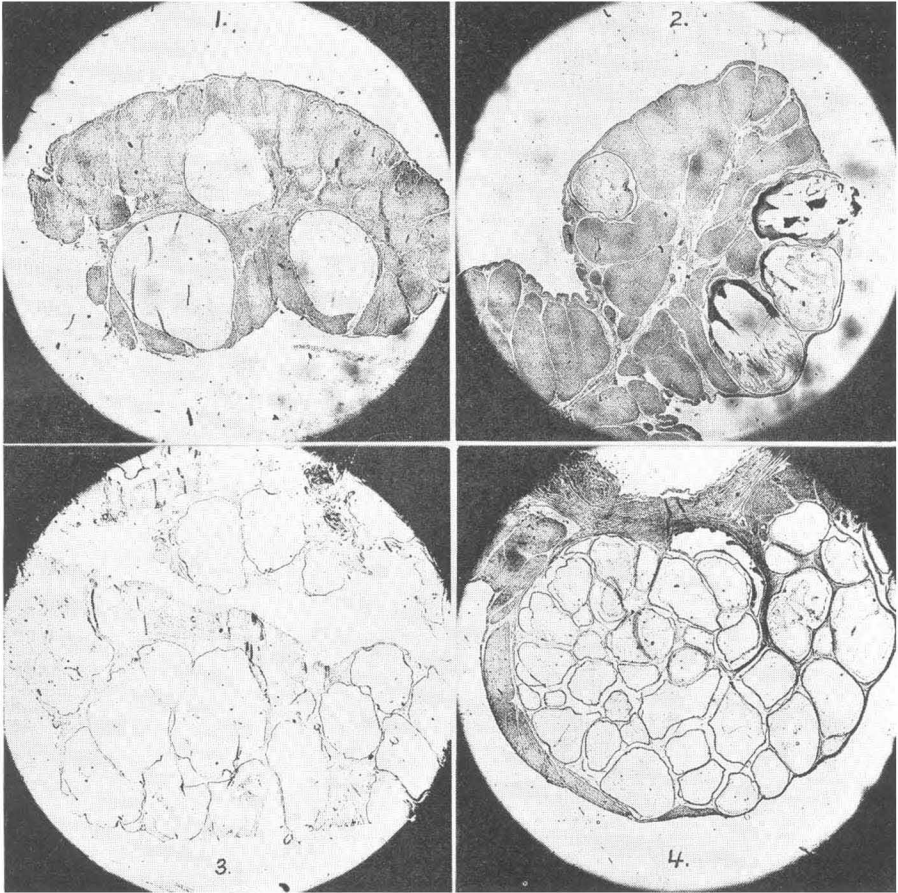
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Contagious diseases are sometimes controlled by taking advantage either of serological tests or of vaccination procedures based on immunology. The immunology of infectious laryngotracheitis has never been worked out. While this study is not exhaustive, progress has been made toward a better understanding of the disease, and a tentative vaccination method for its control is advanced from the laboratory stage to more extensive experiments in the field.

MASSACHUSETTS STATE COLLEGE
AMHERST, MASS.



Traumatic Cysts in the Mucous Membrane of the Bursa of Fabricius

- 1. Cockerel MF-89852**
- 2. Pullet MF-89862**
- 3. Cockerel MF-89879**
- 4. Cockerel MF-89923**

(Photomicrographs by Professor W. H. Davis, Botany Department, Massachusetts State College)

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THE IMMUNOLOGY OF INFECTIOUS LARYNGOTRACHEITIS

By Charles S. Gibbs,
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In undertaking this study it was necessary first of all to determine whether birds could be artificially immunized to infectious laryngotracheitis or not. After having determined this point, and realizing that very little immunological work has been done with birds, it was felt that some preliminary experiments under controlled conditions in the laboratory would yield results more quickly than could be expected in the field. This report shows that this anticipation has been fulfilled and a foundation has been laid for attempting field work in an experimental way as the next step forward in the investigation of this project.

In the experiments reported, attempts were made to immunize birds by inoculating the virus into some portion of the body other than the respiratory tract. Naturally, the exudate and tissues containing the virus had to be modified to facilitate inoculation, and this may have had some effect upon the results, but the general principle of keeping the virus away from the respiratory tract was adhered to as much as possible.

FEEDING EXPERIMENTS WITH UNFILTERED VIRUS

On nine consecutive days, 36 chickens six weeks old were fed infectious laryngotracheal exudate in gelatin capsules without any of them showing symptoms of disease. Nine control chickens from the same flock, inoculated intratracheally with the same virus, contracted the disease and seven of them died. Four weeks after the feeding had been completed, the 36 chickens were inoculated intratracheally with fresh infectious laryngotracheal exudate, and all of them came down with the disease, from which 29 died. This experiment indicates that no lasting immunity was stimulated by feeding chickens six weeks old with virulent infectious laryngotracheitis virus for nine days.

SEITZ FILTERED VIRUS Intravenous Inoculation

1. *Single Inoculations of Diluted Virus*

An attempt was made to immunize birds by inoculation in the wing veins with various dilutions of infectious laryngotracheitis virus which had been passed through Seitz E. K. Schichten filters to remove bacteria. The first part of this project, or the filtrations through the Seitz filters, was the most difficult to control, because no satisfactory way of standardizing the virus had been discovered, and the filtrates could not always be depended upon to contain sufficient virus to produce the desired degree of immunity unless carefully checked by adequate controls. When it was found by means of the controls that the virus did not pass the Seitz filters in sufficient quantity to infect susceptible chickens, inoculated intratracheally, the filtrations and inoculations were repeated with fresh virus until satisfactory results were secured.

Sixty-eight chicks, which had never been exposed to infectious laryngotracheitis, were divided into four groups, each group consisting of fifteen chickens for intravenous inoculation and two for intratracheal inoculation or controls. Each

chicken to be immunized was inoculated in the wing vein with 1 cc. of fresh infectious laryngotracheal exudate which had been diluted with physiological saline and filtered through Seitz filters. In Group 1 the filtrate was diluted 1-10, and in Groups 2, 3, and 4 the dilutions were 1-25, 1-50, and 1-100, respectively. The eight controls were inoculated intratracheally with the filtrate as diluted for each group. The results of the intravenous inoculations are reported in Table 1.

TABLE 1. — THE TITRATION OF INFECTIOUS LARYNGOTRACHEITIS VIRUS FOR INTRAVENOUS INOCULATION

Group	Dilution of virus	Inoculated intravenously			Controls (inoculated intratracheally)		
		Unaffected	Affected		Unaffected	Affected	
			Recovered	Died		Recovered	Died
1	1-10	1	4	10	0	0	2
2	1-25	3	7	5	0	0	2
3	1-50	11	2	2	1	1	0
4	1-100	15	0	0	2	0	0
Totals		30	13	17	3	1	4

A study of Table 1 will show that dilutions 1-10, 1-25, and 1-50 were too strong, since 14 chickens contracted the disease in Group 1, 12 in Group 2, and 4 in Group 3. The 1-100 dilution was too weak, for both controls were unaffected.

All of the chickens surviving the intravenous inoculations were inoculated intratracheally with fresh infectious laryngotracheitis virus. The results are given in Table 2.

TABLE 2.—THE RESULTS OF THE INTRATRACHEAL INOCULATIONS OF THE CHICKENS PREVIOUSLY INOCULATED INTRAVENOUSLY (TABLE 1).

Group	Inoculated intratracheally after intravenous inoculation			Controls (both inoculations intratracheal)			
	Unaffected	Affected		Unaffected	Affected		
		Recovered	Died		Recovered	Died	
1	5	0	0	—	—	—	
2	10	0	0	—	—	—	
3	10	3	0	1	1	0	
4	8	6	1	0	0	2	
Totals		33	9	1	1	1	2

The data in Table 2 show that the four chickens in Group 1 and the seven chickens in Group 2, affected by the intravenous inoculations, were immune to infectious laryngotracheitis. The unaffected chickens in Groups 1 and 2 were immune also. Three of the 11 unaffected chickens in Group 3 contracted infectious laryngotracheitis without fatal termination, while the two affected chickens were found to be immune. Seven of the fifteen chickens unaffected by the intravenous inoculations in Group 4 were affected by the intratracheal swabbings and one died.

One of the control chickens in Group 3, Table 1, was affected by the intratracheal inoculation and the other was not. In Table 2 these chickens are reversed as a result of the intratracheal swabbings. Both of the chickens in Group 4 contracted infectious laryngotracheitis and died, indicating that the virus had been diluted too much, as was suspected.

2. *Intermittent Inoculation of Small Doses of Virus.*

Chickens, six weeks of age, were divided into four groups of 12 each, and inoculated intravenously with tracheal exudate which had been prepared by triturating 1 gram of desiccated tracheal exudate with 25 cc. of saline solution and filtering through a Seitz filter. The chickens in Group 1 were inoculated with 1/4 cc. of the filtered tracheal exudate; those in Group 2, with 1/4 cc. and 1/2 cc. at an interval of seven days; those in Group 3, with 1/4 cc., 1/2 cc., and 3/4 cc. of the same preparation in the same manner; and those in Group 4, with 1/4 cc., 1/2 cc., 1 cc., and 2 cc. Twenty-two controls were swabbed intratracheally and contracted infectious laryngotracheitis and died, while the chickens inoculated intravenously remained unaffected.

Seven days after the final intravenous inoculation the 48 unaffected chickens and two new controls were treated intratracheally with fresh laryngotracheitis virus. The results are shown in Table 3.

TABLE 3. — TESTS OF IMMUNITY IMPARTED TO CHICKENS BY INTERMITTENT INTRAVENOUS INOCULATIONS WITH SMALL DOSES OF VIRUS

Group	Inoculated intratracheally after intravenous inoculation			Controls (intratracheal inoculation only)
	Affected			Died
	Unaffected	Recovered	Died	
1	2	0	10	—
2	4	3	5	—
3	10	2	0	2
4	12	0	0	—
Totals	28	5	15	2

Table 3 indicates that the chickens in Groups 1 and 2 receiving one and two intravenous inoculations of Seitz filtered tracheal exudates, respectively, were not protected against intratracheal swabbings of virulent virus, while the chickens in Groups 3 and 4 receiving increasingly larger doses of the filtrate over longer periods of time were made immune to infectious laryngotracheitis.

The Duration of Immunity

Another flock of chickens as nearly like the preceding as it was possible to secure were treated in a similar manner, except that they were not inoculated intratracheally until a period of four months had passed, in order to allow the immunity time to wear off if it were inclined to do so. The treatments were as follows: Group 1 received 1/4 cc. of the Seitz filtered tracheal exudate diluted 1-25; Group 2, 1/4 and 1/2 cc.; Group 3, 1/4, 1/2, 3/4, and 2 cc.; and Group 4, 1/4, 1/2, 3/4, 2, 2, 3/4, 1/2, and 1/4 cc. The doses were administered at weekly intervals, and the fluid was warmed to body temperature each time. The results are shown in Table 4.

This experiment indicates that chickens may be immunized to infectious laryngotracheitis by inoculation with small doses of properly diluted infectious laryngotracheal exudate in physiological saline solution at seven-day intervals until four or five doses have been given, over a period of twenty-eight days.

Success or failure to secure an enduring immunity by this method depends upon the virulence of the Seitz filtered virus. This virulence may be determined by inoculating controls of the same age and resistance as the birds it is desired to immunize. If at any time the filtrate fails to cause active symptoms of infectious laryngotracheitis in the controls, its value as an immunizing agent should be questioned.

Group 2 was treated in the same manner with 2 cc. of the tracheal exudate solution, diluted 1-25 and filtered through a sterile Seitz filter, and Groups 3 and 4 were inoculated with two and three doses of the same liquid at weekly intervals. Groups 5 and 6 were treated with tracheal exudate which had been diluted 1-25 in saline and glycerol mixed in equal volumes.

TABLE 5. — THE RESULTS OF SUBCUTANEOUS INOCULATIONS OF INFECTIOUS LARYNGOTRACHEITIS VIRUS

Group	Inoculated subcutaneously				Controls (inoculated intratracheally)
	Unaffected	Abscesses	Affected		Died
			Recovered	Died	
1	2	2	3	3	2
2	3	0	4	3	2
3	3	0	3	4	2
4	6	0	3	1	2
5	7	0	2	1	2
6	8	0	2	0	2
Totals	29	2	17	12	12

Table 5 shows that the chickens in Groups 1,2,3, and 4 were not as well protected as those in Groups 5 and 6. Also, it shows that the virus which was inoculated intratracheally was virulent, for all of the controls died. Obviously the degree of immunity imparted by subcutaneous inoculations is relative, depending upon the virulence of the virus, the number of inoculations, and the viscosity of the fluid injected.

Since the farmer with whom the field project was carried on felt that three or more inoculations, involving as many handlings of the birds, was a serious drawback, the study of the subcutaneous method was abandoned as being impractical under range conditions.

TISSUE VACCINES

The success of Boynton (1928), Kelsner (1928), and Nakizaki et al. (1926) with tissues vaccines for rinderpest, the experiments of Laidlaw and Dunkin (1927, 1928) with distemper in ferrets and dogs, and Pasteur's rabies vaccine suggest that possibly a similar biologic might be developed for infectious laryngotracheitis, since Gibbs (1931) and Beach (1931) report finding the virus associated with the tissues of diseased chickens. However, before attempting any vaccinations it was deemed best to approach this project by studying the constancy, the virulence, and the preservation of the virus in the tissues as compared with that in the trachea of the same bird.

By careful manipulation all three points were determined in one experiment as follows:

Twelve chickens, six weeks old, were placed in cages, so that they could be observed and handled better than if allowed to run in a single large room, or colony house. The chickens were inoculated intratracheally with fresh tracheal exudate, and every other day beginning the second day after the inoculation two of the chickens were killed and the liver, spleen, blood, and tracheal exudate removed under aseptic conditions and placed in sterile mortars. The tissues were pounded and rubbed into smooth masses with sterile pestles. Sufficient saline was added to yield a 20 per cent suspension of tissue pulp, and after brief stirring the contents of the mortars were transferred to sterile bottles containing glass beads. The bottles

were shaken vigorously for fifteen or twenty minutes until the fragments were broken up as much as possible. The contents of the bottles were filtered through several layers of sterile cheesecloth into sterile flasks. All of the coarse particles of fibrous tissue, serous and mucous membranes, and trabeculae were thus removed.

Each suspension of tissue was divided into four parts. Part 1 was reserved for control. To Part 2 was added 40 per cent formalin until a final concentration of 0.1 per cent formaldehyde was attained, and the mixture shaken and placed in the ice box for twenty hours before use. A solution of carbolic acid was added to Part 3 until 0.5 per cent phenol was obtained. Glycerol was shaken into Part 4 until a homogeneous mixture was secured, containing about 25 per cent of the fluid. Two chickens six weeks old were inoculated intratracheally with each preparation until the tissues from the twelve original birds on the experiment had been tested and the results noted.

The virus was found in the blood and tissues of four of the chickens soon after external symptoms were noticed, lingered for about 48 hours, and disappeared so that it could not be determined by intratracheal inoculation outside of the respiratory tract.¹ Measured by the comparative mortality of the chickens in the various groups, the virus in the tracheal exudate was more virulent than that in the tissues. Formalin and phenol apparently destroyed the virus in every preparation in which they were used. The glycerolized tracheal exudates maintained their virulence the best of all, but this preparation has already been studied, and it is unnecessary to discuss it further. No evidence of the virus was found either in the tissues or in the blood just before death as so often occurs in bacterial diseases. The chickens on this experiment apparently died of asphyxiation, due to plugging of either the larynx or syrinx by pseudomembrane.

The results of this experiment indicate that it is impossible to prepare satisfactory tissue vaccines for infectious laryngotracheitis by the methods used in this investigation.

HYPERIMMUNE SERUM

Intravenous Inoculations

Hyperimmune serum was prepared by inoculating pullets intravenously with tracheal exudates, which had been diluted 1-10 in saline solution and filtered through Seitz E. K. Schichten filters. Two cc. were inoculated into the wing veins at five-day intervals until six injections had been made. Eight days after the last intravenous inoculation the birds were killed, the blood drawn into sterile cylinders, and the clot allowed to form at room temperature. Later the clot was lifted from the sides of the cylinders by means of sterile rods, and sterile weights were placed on the surfaces of the clotted blood to press the serum out. After standing in the ice box for forty-eight hours the clear serum was pipetted from the cylinders into a sterile bottle, 0.5 per cent phenol added, and the bottle shaken until a uniform mixture was obtained. The serum was stored in the ice box until the chickens were ready for the experiment.

For these experiments 42 pullets and cockerels three months old, which had never been exposed to infectious laryngotracheitis, were secured. They were divided into three groups of 14 birds each, and inoculated intratracheally with infectious laryngotracheitis virus. Then 10 of the chickens in each group were inoculated intravenously with hyperimmune serum, while four were left as untreated controls. The birds in Group 1 received 1 cc. of hyperimmune serum on

¹ In these experiments the virus appeared to be associated with the erythrocytes, and its antibody with the leucocytes and serum. Investigation is under way to determine this point more fully.

the day symptoms appeared; those in Group 2 received 1/2 cc. on the same day and 3/4 cc. on the day following; while those in Group 3 were treated with 1 cc. of hyperimmune serum two days after symptoms appeared. The results of this experiment are tabulated as follows:—

TABLE 6. — THE RESULTS OF INTRAVENOUS INOCULATION WITH HYPERIMMUNE SERUM

Group	Treated with virus and hyperimmune serum		Controls (virus but no serum)	
	Affected		Affected	
	Recovered	Died	Recovered	Died
1	8	2	0	4
2	10	0	1	3
3	2	8	0	4
Totals	20	10	1	11

Table 6 indicates that one large dose of the hyperimmune serum on the day symptoms appear is not as effective as smaller doses given twenty-four hours apart. Serum administered intravenously two days after symptoms have already appeared, or in other words when the disease is pretty well advanced, is of little value. All of the birds protected by the hyperimmune serum and recovering from the intratracheal inoculations were found to be immune a month later. The reaction in these partially immune birds may have been similar to the serum and virus immunization process so often taken advantage of in hog cholera, rinderpest, and sometimes in dog distemper.

The task of inoculating birds intravenously was slow, painstaking labor, because each bird had to be treated individually and the needle carefully maneuvered into the wing vein. For this reason it was almost impossible to inoculate successfully birds under three months of age. Since infectious laryngotracheitis is primarily a disease of chickens, this method may not be practical on a large scale.

Subcutaneous Inoculations

In order to determine the efficacy of the subcutaneous inoculation of hyperimmune serum, 42 pullets and cockerels three months old were divided into three groups of 14 birds each, and inoculated intratracheally with infectious laryngotracheitis virus. Then 10 birds in each group were treated subcutaneously with hyperimmune serum, and 12 controls, 4 for each group, were not given any serum. The doses of serum for each group varied as follows:—

Group 1 received 1 cc. of serum on the day the tracheas were inoculated, and a second dose was given twenty-four hours later on the other side of the breast. Group 2 was inoculated in the same manner, except that the inoculations were commenced the second day after symptoms appeared. Group 3 was given 1 cc. of hyperimmune serum only on the day symptoms of infectious laryngotracheitis appeared.

In these experiments the hyperimmune serum apparently filtered slowly into the tissues and did not neutralize the virus of infectious laryngotracheitis to any great extent. The best results were secured in Group 1 in which chickens were inoculated before symptoms of disease appeared. All of the surviving chickens were found to be immune a month after recovering from the disease, except the controls and two unaffected chickens in Group 1. The controls died, but the two unaffected chickens in Group 1 recovered.

The results of these inoculations are reported in Table 7.

TABLE 7.— THE RESULTS OF SUBCUTANEOUS INOCULATION WITH HYPERIMMUNE SERUM

Group	Treated with virus and hyperimmune serum			Controls (virus but no serum)	
	Unaffected	Affected		Died	
		Recovered	Died		
1	2	4	4		4
2	0	2	8		4
3	0	3	7		4
Totals	2	9	19		12

The Duration of Immunity

A determination of the duration of immunity stimulated by the intravenous inoculation of hyperimmune serum was made by selecting 42 pullets and cockerels and inoculating 30 of them intravenously with hyperimmune serum. The birds were given 1/4 cc. of serum the first day and 3/4 cc. the second. The 12 uninoculated birds were held as controls.

Five days after the serum treatments, 10 inoculated and 4 uninoculated controls were picked out of the flock at random, placed in separate cages, and inoculated intratracheally with fresh virus. Ten and fifteen days after the serum treatments, the second and third groups were treated likewise. The results are noted in Table 8.

TABLE 8.— DURATION OF IMMUNITY IMPARTED TO PULLETS AND COCKERELS BY HYPERIMMUNE SERUM

Group	Treated with virus and hyperimmune serum			Controls (virus but no serum)	
	Unaffected	Affected		Affected	
		Recovered	Died	Recovered	Died
1	9	1	0	1	3
2	6	4	0	0	4
3	1	1	8	0	4
Totals	16	6	8	1	11

This experiment indicates that immunity imparted to pullets and cockerels by hyperimmune infectious laryngotracheitis serum is temporary. It had evidently reached its full strength five days after administration, as indicated by Group 1, but soon after this it apparently began to decline so that at ten days after inoculation 4 of the 10 chickens had contracted the disease, but none died, as shown by Group 2. Fifteen days after inoculation practically all of the immunity imparted by the hyperimmune serum had disappeared, as revealed by Group 3, in which 9 of the 10 chickens contracted the disease and 8 of them died.

VACCINATION IN THE BURSA OF FABRICIUS

Since the introduction of antigens directly into the subcutaneous tissues and blood stream did not yield satisfactory methods for immunizing birds against

infectious laryngotracheitis, it was decided to try the bursa of Fabricius, since this organ is lined with mucous membrane, and is some distance from the respiratory tract. Up to this time it was not known that the bursa of Fabricius was susceptible to inoculation with infectious laryngotracheitis. After this point had been determined, Hudson and Beaudette (1932) published their results on the cloacal inoculation of chickens with infectious laryngotracheitis virus; and after the work reported in this bulletin had been completed, they published their "Experiments on Immunization against Laryngotracheitis in Fowls" (1933). The work is based on the same principle as the experiments reported herein, although the method of attack, field covered, and interpretation of results differ in several important respects. However, Beaudette and Hudson have had some access to field work, and probably speak with authority on the practical value of this method for immunizing birds outside of the laboratory.

The Bursa of Fabricius

The bursa of Fabricius, named after Hieronymus Fabricius, the celebrated Italian anatomist and surgeon (Borden, 1922), is a bulblike organ lying dorsally to the cloaca in the chicken. It is fully developed at the time of hatching, continues to increase in size as the bird grows, and reaches its maximum development between four and five months (Schauder, 1923; Otte, 1928). At this time it averages one inch long, three-fourths of an inch wide, and one-tenth of an inch in thickness in the Rhode Island Red. It occurs in both cockerels and pullets. Involution commences near the time of sexual maturity (Jolly, 1913; Riddle, 1928), and in the course of two or three months it is reduced to a narrow fold.

The wall of the bursa of Fabricius is made up externally of a serosa composed chiefly of white fibrous connective tissue, and an internal mucous membrane lined with epithelial tissue. A layer of circular and longitudinal involuntary muscle tissue is located between the serous and mucous membranes. The mucous membrane and some of the muscle tissues are folded longitudinally so that the organ presents a wrinkled appearance internally. The external appearance is smooth. The aperture into the bursa of Fabricius is small and controlled by a sphincter muscle until involution commences. At the time of retrogression, the sphincter muscle gradually loses its elasticity and the opening enlarges until involution is complete. In the adult bird the opening appears as a horizontal slit leading into the bursal fold.

The physiological function of the bursa of Fabricius has been disputed. Retterer and Lelievre (1913) believed it to be concerned in fertilization. Jolly (1911) ascribed a hematopoietic function to it, and Jolly and Levin (1911) stated that it might be associated with nutrition for it degenerates rapidly during starvation, and is abundantly supplied with lymphatics.

Infection of the Bursa of Fabricius with the Virus of Infectious Laryngotracheitis

In starting these experimental studies on the bursa of Fabricius inoculation, it was necessary first of all to determine whether this organ could be infected with the virus of infectious laryngotracheitis, and if so, how long the virus would remain in it.

Twenty-four chickens three months old were selected for this study. Twelve of them had never been exposed to the disease, and the others had been immunized by the intravenous method. All of them were inoculated in the bursa of Fabricius with infectious laryngotracheitis virus, which had been prepared in the following

manner. Desiccated virus was ground up in a sterile mortar with sufficient sterile saline solution to make a thin paste. A pinch of cotton was twisted on the end of a wooden applicator to make a swab that would easily pass through the opening leading into the bursa of Fabricius. The swab was dipped into the paste, and gently pushed through the bursal orifice of each bird and the mucous membrane carefully swabbed, taking care to get between the folds. Beginning the third day after inoculation, a swab was inserted into the bursa of each bird and some of the exudate removed and re-inoculated into the tracheas of chickens which had never been exposed to infectious laryngotracheitis. The results of this experiment are reported in Table 9.

TABLE 9. — THE NUMBER OF DAYS THE VIRUS WAS RECOVERED FROM THE BURSA OF FABRICIUS

Days	Unimmunized birds	Immune controls
1	12	8
2	12	5
3	12	2
4	12	0
5	11	0
6	8	0
7	5	0
8	3	0
9	2	0
10	0	0

In this table the unimmunized birds refer to the 12 chickens which had never been exposed to infectious laryngotracheitis until they were vaccinated in the bursa of Fabricius, and the immune controls are the ones which had been previously immunized by the intravenous method. This experiment indicates that the bursa was infected in the unimmunized birds and was not infected in the immune controls. Evidence of this was further demonstrated on physical examination of the cloaca and the opening into the bursal orifice. On the third day after vaccination, the bursal orifice and sometimes the cloaca were inflamed and swollen in the unvaccinated birds, while these parts in the immune controls remained practically normal.

Also this experiment indicates that the virus was retained in the bursa of Fabricius of the unimmunized birds for five days after vaccination, before it began to disappear, and it was demonstrated in some of the birds up to the ninth day. The virus in the bursa of the immune controls apparently began to deteriorate at once and could not be demonstrated four days after inoculation.

Two weeks after all visible inflammation and swelling of the bursal orifice had ceased, the chickens were inoculated intratracheally with infectious laryngotracheitis virus and were found to be immune. Four controls inoculated at the same time contracted the disease and three died.

After it had been established that the bursa of Fabricius could be infected with the virus of infectious laryngotracheitis, and that the infection was apparently of sufficient duration to stimulate the production of immune bodies which reached the mucous membrane of the trachea, it was decided to immunize birds on a comparatively large scale, under laboratory conditions, and to determine the duration of immunity established by this method more fully, as well as to study any other complications which might arise. No special refinements were made in the preparation of the vaccine, except that the viruses used had been prepared by the Swift method (Gibbs, 1933a), and had killed chickens one month old in a week. This arbitrary standard seemed to be necessary, because it was observed in preliminary experiments that the bursa of Fabricius was not as easily infected as the larynx and trachea, so that any virus of diminished virulence might not produce

satisfactory immunity, but lead to erroneous conclusions in regard to the efficacy of the method.

The Vaccination of Roosters and Fowls

A mixed group of birds was available for this experiment, consisting of 12 roosters and 23 fowls. All of these birds had reached maturity and the bursa of Fabricius had completely involuted. The birds were placed in individual cages, and 27 of them inoculated in the bursal fold, while 8 controls were inoculated intratracheally. Eight of the inoculated birds came down with infectious laryngotracheitis in five days, which is about the average incubation period for fowls in production, and two of them died. Seventeen days after vaccination the birds were inoculated intratracheally with infectious laryngotracheitis virus, and all were found to be immune, except the controls. The controls contracted the disease and three died.

The surviving birds were held under observation for twenty-five days without any further indications of disease, except that one of the vaccinated fowls developed proctitis and inflammation of the oviduct, in addition to infection of the bursa of Fabricius. This condition led to prolapse of the oviduct. If this bird had been in a flock instead of in an individual cage, she would undoubtedly have been picked to death and the fatality charged to cannibalism without the real facts of the case being known.

The Vaccination of Pullets and Cockerels

In this experiment the pullets and cockerels were divided into four groups, according to age, 200 being vaccinated in the bursa of Fabricius while 82 were held in separate cages as controls. Fourteen days from the date of vaccination all of the birds, including the controls, were inoculated intratracheally with infectious laryngotracheitis virus. The results of this experiment are summarized in Table 10.

TABLE 10. — TESTS OF IMMUNITY OF PULLETS AND COCKERELS FOLLOWING BURSAL VACCINATION

Group	Age (Days)	Inoculated intratracheally after vaccination				Controls (intratracheal inoculation only)		
		Affected				Affected		
		Unaffected	Recovered	Died	Cannibalism	Unaffected	Recovered	Died
1	14	38	5	0	0	1	10	6
2	10	47	3	5	1	0	4	12
3	6	31	2	6	0	0	18	21
4	12	59	0	2	1	0	4	6
Totals		175	10	13	2	1	36	45

In Group 1, as indicated in Table 10, all of the vaccinated birds were protected, except five which apparently infected themselves soon after inoculation, and had mild attacks of infectious laryngotracheitis. It would appear natural for some of the birds to infect themselves before sufficient immunity was developed for protection, either by picking the vaccinated parts or from contaminated droppings on the floor. Of the 17 controls, 1 was found to be naturally immune, and 16 contracted the disease from which 10 recovered and 6 died. The birds were held for forty days after vaccination and none of them showed any after effects of the treatment.

Of the 55 vaccinated birds in Group 2, 9 contracted the disease after the usual incubation period for infectious laryngotracheitis in chickens, and of these 1 was killed as a result of cannibalism, 5 died naturally, and 3 recovered. When tested intratracheally for immunity, the 50 vaccinated birds living were found to be immune, but the 16 controls contracted the disease from which 12 died and 4 recovered. The surviving chickens were held for thirty-three days and appeared to be developing normally.

At necropsy 9 vaccinated chickens and 10 controls showed lesions of coccidiosis, and the mortality in both the vaccinated and control groups was confined almost entirely to the birds suffering from this complication. This experiment indicates that birds having chronic coccidiosis are not good risks for bursal vaccination with infectious laryngotracheitis virus.

In Group 3, 31 of the vaccinated birds were immune and 8 contracted the disease, of which 6 died and 2 recovered. All of the controls developed infectious laryngotracheitis and 21 died while 18 recovered. Lesions of coccidiosis were evident at necropsy in the 8 vaccinated birds showing symptoms of infectious laryngotracheitis and 11 of the controls. Once more the fact was forced upon the investigator that birds weakened by coccidiosis are not good subjects for treatment by this method.

Three days after vaccination two of the birds in Group 4 showed clinical symptoms of infectious laryngotracheitis and died. A pullet in which the cloaca became infected as well as the bursa, died of cannibalism. Of the 62 birds vaccinated, 59 did not show any symptoms of disease. All of the controls contracted infectious laryngotracheitis and six died, while four recovered.

Traumatic Cysts

Four of the vaccinated birds, one pullet and three cockerels, had traumatic cysts in the bursa of Fabricius similar to those shown in the photograph. The cysts looked like tapioca embedded in the mucous membrane, from which they readily shelled out, leaving small cavities. When placed in cold water the cysts swelled up and burst, liberating a sticky gelatinous mass, which gradually went into solution, while the covering membrane remained broken but insoluble in water. The gelatinous mass was precipitated from the solution by the addition of a few drops of 1 per cent acetic acid or alcohol and redissolved in 20 per cent NaOH solution, but it was not redissolved by an excess of acid. The covering membranes were not soluble in any of the reagents used. These tests indicate that the traumatic cysts consist largely of mucous.

The significance of these cysts is not fully realized at present. The vaccination must have had something to do with them for they have not been found in unvaccinated birds.

The Duration of Immunity

Fifty-six chickens from Group 4, immune to infectious laryngotracheitis at the time of placing in a colony house, were kept for ten months for studies on the duration of immunity. One of the cockerels died of avian paralysis before the tests were commenced. However, five months after vaccination 55 of the birds were inoculated intratracheally with infectious laryngotracheitis virus, and 6 of them came down with clinical infectious laryngotracheitis. Three of the infected birds died, three recovered, five developed laryngotracheal rales, and two proved to be chronic carriers. None of the 52 living birds were found to be harboring the

virus in the bursa of Fabricius, although the bursal fold was closed with adhesions in 2 of the roosters so that they could not be tested for the carrier stage. One pullet died before the experiment was closed. Necropsy revealed the bursal fold constricted so that the opening from the oviduct into the cloaca was obstructed, and the bird could not lay. Two completely formed eggs were found in the oviduct.

Bacteria in Tracheal Exudates Complicate Bursal Vaccinations

In order to study the importance of bacteria in bursal vaccinations, 36 chickens six weeks old were divided into three groups of 12 each. Group 1 was inoculated with Seitz-filtered infectious laryngotracheitis virus, Group 2 with streptococci isolated from the tracheas of chickens and fowls (Gibbs, 1933b), and Group 3 with staphylococci from the tracheas of normal fowls which showed pathogenic properties when the mucous membrane was injured as in infectious laryngotracheitis (Gibbs, 1930). The same degree of infection occurred in Groups 1 and 2, and in three of the chickens in Group 3, so that it was impossible to distinguish the bacterial from the virus inoculation or "take." It was concluded from this experiment that bacteria in the tracheal exudate may complicate vaccinations in the bursa of Fabricius and interfere with the determination of successful takes. For this reason it was felt that considerable care should be exercised in the selection and cultivation of tracheal exudate for vaccine.

In the routine preparation of infectious laryngotracheal exudate for vaccine, the virus should be filtered in order to free it from bacteria. It should always be prepared from chickens one month old, which have been hatched in incubators and reared under especially sanitary conditions away from all other birds.

The filtration of infectious laryngotracheitis virus presents some difficult problems. However, if a modification of Ward's (1928) method is used the virus should pass Seitz and Berkefeld filters more readily than if the exudate is triturated in physiological saline solution or yeast water alone. In applying the modified Ward method to the filtration of infectious laryngotracheitis virus, the exudates were ground up in mortars with fragments of Pyrex glass, and suspended in hormone broth. Before pouring the mixture into the filters the surfaces of the Seitz disks were covered with a layer of broken glass. Extra fragments of glass were not added to the Berkefeld filters because they could not be held in place before pouring in the exudate and starting the suction. The hormone broth is a modification of Huntoon's method, and it was prepared as follows:—

Four hundred grams of minced chicken heart free from fat, ten grams of pepsin, and five grams of salt were boiled for fifteen minutes in one liter of water. The meat particles were removed by straining through cheesecloth, and the broth autoclaved at 15 pounds extra pressure for half an hour. It was allowed to stand over night in the ice box. The next morning the supernatant fluid was decanted off, 25 cc. to a flask, and autoclaved for 15 minutes.

Table 11 shows the results of this experiment with Seitz and Berkefeld V, N, and W filters, and indicates that hormone broth plus Ward's method of filtration is more efficacious than either physiological saline solution or yeast water alone. If this method of filtration had been known when the experiments on the intravenous and subcutaneous inoculation of infectious laryngotracheitis were made, the results might have been more encouraging. Discrepancies of this sort are to be expected in a project which has not passed out of the experimental stage.

TABLE 11. — FILTRATION OF INFECTIOUS LARYNGOTRACHEITIS VIRUS

Dilution of Filtrate	Saline				Yeast Water				Hormone Broth			
	S	V	N	W	S	V	N	W	S	V	N	W
1-10	0	0	0	0	0	+	0	0	+	+	+	0
1-25	0	+	0	0	+	+	0	0	+	+	+	+
1-50	+	+	0	0	+	+	+	0	+	+	+	+

Legend:—S - Seitz filter
V - Berkefeld V filter
N - Berkefeld N filter
W - Berkefeld W filter

CONTINUOUS CULTIVATION OF THE VIRUS IN THE BURSA OF FABRICIUS

This part of the project was designed to determine whether the virus of infectious laryngotracheitis could be continuously cultivated in the bursa of Fabricius or not, and if so, whether bacteria interfering with vaccinations could be eliminated or not. Furthermore, it was deemed important to study the immunizing properties of the viruses as they were being continuously cultivated, in the hope that they would become adapted to growth in this organ and lead to the discovery of a more satisfactory immunizing agent than has been found up to the present.

Accordingly, two viruses which had given the best results in previous bursal vaccinations were selected for this study. In each of these trials two chickens were inoculated in the bursa of Fabricius and the virus was transferred to the same organ of two other chickens every three days. At each transfer two control chickens were inoculated intratracheally with the same virus and the results recorded as indicated in Tables 12 and 13.

TABLE 12. — CONTINUOUS CULTIVATION OF VIRUS X IN THE BURSA OF FABRICIUS

Date	Bursa	Trachea	Result	Immunity
8/27/32	-	V-1204	Unaffected	+
	-	V-1205	Died	-
8/30/32	V-1206	V-1208	Unaffected	+
	V-1207	V-1209	Died	-
9/ 3/32	V-1210*	-	-	-
	V-1211*	-	-	-
9/ 6/32	V-1212	V-1214	Unaffected	0
	V-1213	V-1215	Unaffected	0
9/ 9/32	-	V-1216	Recovered	+
	-	V-1217	Recovered	+
9/12/32	V-1220	V-1222	Unaffected	0
	V-1221	V-1224	Died	-
9/15/32	V-1225	V-1227	Died	-
	V-1226	V-1228	Died	-
9/19/32	V-1229	V-1231	Died	-
	V-1230	V-1232	Recovered	+
9/22/32	V-1233	V-1235	Unaffected	0
	V-1234	V-1223	Unaffected	0

*Died

TABLE 13. — CONTINUOUS CULTIVATION OF VIRUS Y IN THE BURSA OF FABRICIUS

Date	Bursa	Trachea	Result	Immunity
10/18/32	—	V-1236	Died	—
	—	V-1237	Died	—
10/24/32	—	V-1238	Died	—
	—	V-1239	Recovered	+
10/25/32	V-1242	V-1244	Unaffected	+
	V-1243	V-1245	Died	—
10/28/32	V-1249	V-1251	Unaffected	+
	V-1250	V-1252	Died	—
10/31/32	V-1255	V-1259	Died	—
	V-1256	V-1260	Died	—
11/ 3/32	V-1257	V-1262	Died	—
	V-1258	V-1264	Died	—
11/ 6/32	V-1261	V-1266	Died	—
	V-1263	V-1268	Died	—
11/ 9/32	V-1265	V-1270	Died	—
	V-1267	V-1296	Died	—
		V-1297	Died	—
11/12/32	V-1269	V-1273	Died	—
	V-1271	V-1275	Died	—
11/17/32	V-1272	V-1277	Died	—
	V-1274	V-1279	Unaffected	+
11/19/32	V-1276	V-1281	Unaffected	+
	V-1278	V-1283	Recovered	+
11/23/32	V-1280	V-1285	Unaffected	0
	V-1282	V-1287	Died	—
11/26/32	V-1284	V-1289	Unaffected	0
	V-1286	V-1291	Died	—
11/29/32	V-1288	V-1293	Unaffected	0
	V-1290	V-1295	Died	—
12/ 2/32	V-1292	V-1297	Recovered	+
	V-1294	V-1299	Died	—
12/ 6/32	V-1296	V-1301	Died	—
	V-1298	V-1303	Died	—
12/ 9/32	V-1300	V-1305	Unaffected	+
	V-1302	V-1307	Recovered	+
12/12/32	V-1304	V-1308	Recovered	+
	V-1306	V-1309	Died	—

Virus X, Table 12, was not very satisfactory because it was lost twice, once in chickens V-1212 and V-1213, and again in chickens V-1233 and V-1234. Virus Y, Table 13, was cultivated continuously in the bursa of Fabricius for 58 days without loss of virulence, or change in immunizing properties. The bacterial flora was different than in viruses from the respiratory tract. The bacteria were of intestinal origin and did not influence the results in vaccinations, but further study is needed to clear up this point. This study was not continued further at this time because no advantage was obtained in cultivating the virus exclusively in the bursa of Fabricius, since a better check on the virulence was maintained when the chickens were inoculated intratracheally.

However, in selecting a virus for vaccine in the future, virus Y will be chosen rather than virus X, because of its uniform results in this experiment.

CLOACAL VERSUS BURSAL VACCINATION

Forty chickens, three and one-half months old, were divided into two groups of twenty each. Group 1 was inoculated in the cloaca and Group 2 in the bursa of Fabricius. In Group 1, 8 of the chickens developed mild inflammation of the cloaca, which indicated 40 per cent successful takes; while 18, or 90 per cent, of those inoculated in the bursa of Fabricius showed definite indications of takes. Two chickens from each group contracted infectious laryngotracheitis and died. When tested for immunity fourteen days later, 10 of the chickens in Group 1 contracted the disease and 6 died, while all of the 18 living chickens in Group 2 resisted infection.

This experiment indicates that the number of takes is greater when the bursa of Fabricius is inoculated than when the cloaca is swabbed with the same virus in the same manner, and the resulting degree of immunity is greater in the former than it is in the latter case.

DISCUSSION

In attempting immunization in infectious laryngotracheitis the object should be to produce an immunity at least as good as that which follows the disease when naturally contracted. At present this can not be accomplished artificially. Since birds infected in the larynx and trachea are liable to die of asphyxia, due to plugging of the respiratory tract with pseudomembrane, the infection should be kept from this system, unless partial immunity has been previously produced by means of properly filtered tracheal exudate or hyperimmune serum. Laryngotracheitis is primarily a disease of the mucous membranes and infection is not induced by inoculation of the virus into the subcutaneous tissues or blood stream. However, when a relatively large dose of the virus is released in the blood stream, the infectious agent may be carried to the larynx and trachea and a natural attack result. On the other hand, when the virus is introduced into the blood stream in relatively small quantities over a comparatively long time, active immunity is produced. It seems to make little difference whether the small quantities are continuous, as they probably are in the bursa of Fabricius infection, or intermittent as in intravenous inoculation.

Naturally acquired immunity in infectious laryngotracheitis is permanent, while artificially acquired immunity is relative and appears to depend upon the severity of the reaction. Hence the introduction of filtered tracheal exudates into the blood stream, or the inoculation of a mucous surface outside of the trachea such as the bursa of Fabricius, stimulates various degrees of active immunity. The inoculation of hyperimmune serum into the blood stream produces passive immunity only, because the antigen is not present, and the tissues of the bird are not stimulated to produce antibodies. It is impossible to control the reaction in any of the methods suggested for the immunization of birds against infectious laryngotracheitis, because a standard vaccine has not been produced, and in spite of the arbitrary standard suggested in this bulletin for inoculation in the bursa of Fabricius, the danger of spreading the infection where it does not already exist is involved.

Birds weakened, as in coccidiosis, are liable to develop acute infectious laryngotracheitis when vaccinated in the bursa of Fabricius and relatively large numbers die. The simultaneous vaccination of birds against infectious laryngotracheitis and chicken pox was not attempted in these studies, because the experiments were conducted in the laboratory, and it was not considered expedient to make the two tests under such limitations, for fear that the results might

lead to erroneous conclusions. This point should be made clear by carefully controlled experiments under range conditions, and moreover, further studies of infectious laryngotracheitis are needed to determine the practical value of the laboratory methods and immunological theories advanced herein under field conditions, without complication with chicken pox or any other disease which might lessen vitality.

In vaccinating a large number of birds, various degrees of reactions are bound to occur. Some cases will be so mild that only temporary immunity results, while others will respond abnormally and develop a disease so severe that some will die and carriers are produced in a few of those that recover. However, evidence is lacking to show that sufficient virus passes into the blood stream from the infected bursa of Fabricius to be responsible for clinical cases, although it has been demonstrated that birds do infect themselves after vaccination by picking and rubbing the affected parts, and probably indirectly from the virus in the droppings. Thus the danger of creating fresh centers of infection, through the agency of vaccinated birds and carriers, is obvious, in spite of the fact that most of the birds may respond normally and be satisfactorily immunized against the disease.

SUMMARY

1. In order to be acceptable to the majority of poultrymen, treatments for infectious laryngotracheitis must be cheap, easy to administer, and fairly effective.

2. Since the intravenous and subcutaneous methods of stimulating immunity did not measure up to this standard, they are not recommended for field practice.

3. However, the intravenous and subcutaneous studies were conducted under controlled laboratory conditions and the following fundamental immunological points discovered:—

(a) Birds can be immunized against infectious laryngotracheitis by introducing small doses of virus intermittently into some portion of the body other than the respiratory tract.

(b) The virus is carried by the blood and may stimulate the production of immune bodies in all tissues directly in touch with the vascular system.

(c) Large quantities of virus released into the blood stream at once may be carried to the respiratory tract and lead to the development of active symptoms.

4. The inoculation of the bursa of Fabricius appeared to be the most satisfactory way of utilizing the immunological discoveries brought out in this study for immunizing birds against infectious laryngotracheitis.

5. The best time to vaccinate appeared to be between three and four months of age, or when the bursa of Fabricius had reached its greatest development.

6. Only birds in the best of health are suitable for vaccination against infectious laryngotracheitis.

7. The significance of certain traumatic cysts, blow-outs, and adhesions found in the bursa of Fabricius and adjacent parts of some of the birds after vaccination is not understood, in view of the fact that special care was taken to avoid rough handling.

8. Special care should be exercised in selecting and cultivating tracheal exudate for bursa of Fabricius vaccination in order to exclude pathogenic bacteria and viruses other than the causative agent in infectious laryngotracheitis.

9. Infectious laryngotracheitis tracheal exudates desiccated and preserved by the modified Swift method maintain their virulence for several months.

10. Final judgment should be withheld on the bursa of Fabricius vaccination until carefully controlled experiments are completed to determine its practical value under range conditions, and the duration of immunity in fowls in heavy production.

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