The purpose of this paper is to describe the characteristics of an infectious agent isolated from cases of tendovaginitis or arthritis of chickens. This chronic disease of chickens is characterized in the gross by weakness, emaciation, and anemia. A moderately turbid to caseous exudate is found within and around the synovial tissues of the tendons. This material draining into the distal joints gives rise to bursitis and arthritis. The most obvious lesions are enlargement and puffiness of the hock and wing joints, although most of the movable joints, including the mandibular articulation, may be involved. The liver and spleen may show a slight increase in size.

In artificial transmission studies and in field cases, the first sign of infection is a paling of the comb and general depression. The bird will develop an unsure and wobbly gait, and a preference for sitting. An exudate distends the area around the hock and wing joints. Finally a greenish diarrhea develops. Extreme emaciation occurs even though the birds eat up until the time of death. This whole cycle may start as early as 5 days after infection, terminating in death approximately 3 to 4 weeks later. Some few may survive the disease; however, joint lesions remain permanently. In field outbreaks, from 5 to 30 per cent may be affected and of those affected a high percentage die. It has been seen in a number of different breeds and age groups, the youngest being 5 weeks and the oldest being 4 months. It is suspected that a diseased flock, regardless of obvious morbidity, will be generally unthrifty, resulting in a large number of culls and birds of poor carcass quality.

This disease has come to the attention of poultry pathologists only recently. Judging from the increase in frequency and distribution (1–4) since the time of first awareness, the disease promises to be an important economic factor in the poultry industry.

* Present address: School of Agriculture, North Carolina State College, Raleigh.
† McLaughlin Fellow in Infectious Diseases and Immunity, Department of Bacteriology, University of Texas, Medical Branch, Galveston.
Materials and Methods

Bacto-heart infusion broth served as a diluent for all infectious material. The following media were used in attempting to grow bacteria and pleuropneumonia-like organisms (PPLO) ⁴:

- Bacto-blood agar with 5 per cent bovine blood
- Bacto-heart infusion agar with 1 per cent bacto-PPLO serum fraction
- Bacto-heart infusion agar with 1 per cent PPLO serum fraction and 20 μg./ml. DNA (National Biological Company) ⁵
- Bacto-heart infusion agar with 1 per cent PPLO serum fraction, 20 μg./ml. DNA, 1 per cent bacto-yeast autolysate, and 1 per cent bovine serum fraction B-II ⁶

All batches of heart infusion agar and broth were adjusted to pH 7.8 with NaOH prior to autoclaving. Aerobic as well as some anaerobic cultures were incubated at 37°C. Usually joint exudates and egg passage fluids were either placed in the broth, incubated for 3 days, and transferred to a plate or cultured directly on a plate. After 3 days incubation, the plate was examined for bacterial colonies and with the aid of 100 magnification for colonies of PPLO. A slightly different procedure was used for the 16th egg passage of strain 909. In this instance, infectious allantoic-amniotic fluid of the 16th egg passage of strain 909 was blind-passed in broth 5 times at 3 day intervals using 0.5 ml. of inoculum each time. At each passage, a loopful of broth was streaked on a plate, incubated, and examined as above for PPLO and bacteria. Two strains of PPLO that were isolated from cases of chronic respiratory disease of chickens ⁷ were used to check the growth-supporting capacity of the above media for PPLO. These exudates and egg passage fluids were also examined by dark field, and stained with Giemsa or Macchiavello stains for light microscopy.

Strains.—The following strains of the tendovaginitis agent were used: 909, 1071, 1464-29 (mucus), 1464-29 (hock), and Lombardi. Strains 909 and 1071 were isolated by the authors; strains 1464-29 (hock) and 1464-29 (mucus) were received from Dr. N. O. Olson of the University of West Virginia; and strain Lombardi was received from Dr. V. J. Yates of the University of Rhode Island. Strain 909, the first isolated, was repeatedly passed in embryonated eggs so that the characteristic behavior of this type of agent might be learned. Each of these 5 egg-propagated strains was capable of causing symptoms indistinguishable from those observed in field cases of this disease or from the disease caused by passing joint exudates from bird to bird. These various strains were maintained by serial passage in developing chick embryos, or by freezing at −20 or −70°C. Unless otherwise stated, all manipulations were performed with 0.25 ml. of inoculum injected into the yolk sac of 5- to 7-day-old developing chick embryos. The pre- and postinoculated embryos were incubated in the same hatcher at 37 to 39°C. Eggs were candled each morning and fluids were harvested from the dead eggs. Any embryos dying within 72 hours after inoculation were assumed to have died from trauma and were not considered in the tabulations.

Calculations.—For the purpose of comparison, data were expressed either as LD₉₅’s (8) or as the mean day of death (MDD). All experiments were terminated 12 days after infection. Any embryos alive on the 12th day were considered to have died on the 13th day. In order to discern the degree of protection that various drugs afforded, the mean day of death of the untreated control eggs was calculated and this value was subtracted from 13.0 (the maximum possible day of death as defined in our experiments). The difference was, therefore, the maximum possible prolongation of life (MPPL) ² or 100 per cent protection. The efficacy of the various dosages of drugs was evaluated by subtracting the mean day of death of the uninfected controls from the mean day of death of the treated eggs; this difference, the prolongation of life by the drug, was divided by the maximum possible prolongation of life, multiplied by 100, to equal per cent protection achieved by the drug; i.e., (MDD of treated eggs minus MDD of control eggs/MPPL) × 100 = per cent protection (9).

Route of Inoculation.—In order to find the most effective route of inoculation, strain 1071

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1 PPLO, pleuropneumonia-like organisms.
2 MDD, mean day of death.
3 MPPL, maximum possible prolongation of life.
(egg passage 3), and strain Lombardi (egg passage 9) were diluted 1:5 and injected into yolk sac, amniotic sac, and the chorioallantoic membrane. It was necessary that embryos no older than 10 days be used for the amniotic, and chorioallantoic membrane route since the long incubation period prior to mortality confused the results obtained with older embryos. Eight eggs were used for each route. Comparisons in the efficacy of the various routes were made on the basis of mean day of death.

**Filtration.**—A pool of amniotic-fluid harvested from the 11th embryo passage of strain 909, clarified by centrifugation at 2,000 r.p.m. for 5 minutes, was filtered through a 5 and 3 sintered glass filter in order to learn if this agent could pass through a bacteria-retaining filter. Four eggs per each 10-fold dilution were used in determining the LD50 titer of the pre-and postfiltered fluid.

**Antibiotic Sensitivity Spectrum.**—Solutions of terramycin-HCl, chloromycin-HCl, and penicillin G were used for determining the antibiotic sensitivity spectrum of this tendovaginitis agent. The terramycin, aureomycin, and achromycin were prepared as stock solutions of 20 mg./ml. of distilled water. The pH of the solutions was adjusted to 9.0 with NaOH. Sterilization was accomplished by filtration through a 5 and 3 sintered glass filter. The stock solution of 200 mg./ml. of chloromycin was prepared in 95 per cent ethyl alcohol and no further sterilization was necessary. Penicillin and dihydrostreptomycin were rehydrated in sterile vials with sterile distilled water. Either a freshly prepared solution of antibiotic or a frozen stock thawed only once was used for determining the drug's effectiveness. In the titrations, the various concentrations of drug were injected into the yolk sac of the eggs within 30 minutes after the infecting inoculum. Limited facilities necessitated separate titrations of antibiotics; i.e., each antibiotic was titrated on a different day (except for terramycin and chloromycin which were titrated simultaneously using the same inoculum). Therefore, each determination had the following controls: an infected untreated control (equal volume of distilled water replaced the antibiotic injection); an uninfected antibiotic control (broth inoculum followed with the greatest amount of antibiotic that was used in the determination); and in the case of chloromycin, an ethyl alcohol control (infected eggs injected with ethyl alcohol equal in amount to that contained in the lowest dilution of chloromycin). The inoculum for these sensitivity tests was a 0.25 ml. of 1:100 dilution of pooled yolk harvested from the 10th passage of strain 909. This material was kept frozen at -70°C. until use. A 1:100 dilution was chosen because previous titrations of this material showed this dilution to be the maximum dilution that consistently produced 100 per cent mortality. This amount was equal to about 40 LD50's. The amniotic-fluid from this same harvest had approximately the same titer and was also frozen at -70°C. for later use as an inoculum for tissue cultures. The other strains; 1071 (3rd egg passage), 1564-29 mucus (14th egg passage), 1646-29 hock (20th egg passage), and Lombardi (7th egg passage) were tested for their sensitivity to 1 mg./egg of terramycin, aureomycin, dihydrostreptomycin, and 1000 units/egg of penicillin. The inoculum was a 0.25 ml. of a 1:5 dilution of the yolk-amniotic fluid harvested from embryos that died the day of the test. Each strain was run on a separate day, and each had an infected untreated control. In all the determinations involving antibiotics, 8 eggs were used per each antibiotic dilution or strain.

**Electron Microscopy of Strain 1071.**—Crude purification of amniotic-fluid for electron microscopy was accomplished by the following procedure. 40 ml. of amniotic-fluid harvested from eggs that died 5 to 6 days post inoculation (total embryonic age of 12 to 13 days) was aseptically centrifuged for 5 minutes at 2,000 r.p.m. in order to remove large particles. The supernatant was then subjected to a centrifugal force of 25,000 G for 60 minutes. This resulted in a supernatant that was saved, and for purposes of further puri-

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4 We thank Chas. Pfizer and Co., Incorporated for the terramycin, Lederle Laboratories for achromycin, aureomycin, and Parke Davis & Company for the chloromycin.
fication, a sediment that was resuspended in 40 ml. of m/30 phosphate buffer pH 7.2. This low speed, high speed cycle was repeated 3 more times. The sediment resulting from the last high speed centrifugation was resuspended with 1 ml. of 1 per cent formalin in distilled water. This formalin-fixed preparation was placed on collodion films on copper screens and examined with the Philips electron microscope. 40 ml. of normal allantoic-amniotic fluid harvested from the same lot of eggs incubated for 12 to 13 days, killed by refrigeration, was handled in exactly the same manner as the 40 ml. of infected allantoic-amniotic fluid. This was the uninfected control. In order to learn whether the infecting particles were being sedimented by 25,500 G for 60 minutes, LD₅₀ titers were determined for the supernatant and sediment resulting from the first high speed, low speed cycle. The sediment was resuspended in 1 ml. of diluent and the required amount was removed for titering. The broth diluent for the 10-fold dilutions contained 500 units/ml. penicillin. Six eggs were used for each dilution.

**Tissue Cultures.**—Roller tube tissue cultures consisted of 0.5 square cm. explants of 10 to 12 day embryonic chicken heart embedded in a chicken plasma clot and bathed with a medium containing a balanced salt solution, EE₅₀,¹ and chicken serum. These tubes incubated for 4 days at 37°C. had sufficient outgrowth to be infected with a drop of allantoic-amniotic fluid from the 10th passage of strain 909. This drop was introduced in the media change. Usually 4 days' incubation after infection was required before a cytopathogenic effect was noticed in the fibroblasts. At that time, the explants and fibroblasts were stained with May-Greenwald Giemsa in order to emphasize better the effect for photography. There were non-infected controls and controls that had the same infecting dose except the allantoic-amniotic fluid had been heated at 55°C. for 30 minutes.

**Host Range.**—Virulent yolk consisting of a pool of strain 1071, 1464-29 (hock), and Lombardi was used to determine, in a limited sense, the host range of this agent. 0.5 ml. of undiluted yolk was injected intravenously into 4 white mice, 0.03 ml. intracranially into 4 white mice, 1 ml. intraperitoneally into 2 young pigs, and 1 ml. intramuscularly into 2 guinea pigs. Yolk sacs harvested from eggs dying from infection with the 20th passage of strain 909 were ground with alumdum, diluted 1:5, and injected into white mice. Two mice received 1 ml. of the yolk sac suspension intravenously and one mouse received 1.5 ml. intraperitoneally. This 20 per cent yolk sac suspension had a chick embryo LD₅₀ of 10⁻⁴.⁷

**RESULTS**

Colonies of PPLO or bacteria were not detected after repeated culturing of joint exudates and chick embryo passage fluids, using different techniques on a variety of enriched media. Bacteria were not observed when these same fluids were stained with Giemsa and Macchiavello stains and examined at a magnification of 980 with the light microscope. On occasion, small (0.5 μ) intracellular and extracellular red bodies were seen in the Macchiavello stains of hock exudate fluids; and in liver, and amniotic sac impressions of infected chick embryos. Darkfield examinations of these various fluids and exudates revealed no shapes or sizes characteristic of bacteria or spirochetes.

**Embryo Mortality Pattern.**—Repeated egg passage of the various strains via the yolk sac of 5- to 7-day-old embryos revealed the following characteristic mortality pattern: in general, 0.25 ml. of a 1:10 dilution of yolk or allantoic-amniotic fluid harvested from the earlier dying embryos killed 100 per cent of the embryos of the next passage 4 to 12 days after the infecting dose. Most

¹ EE₅₀ is an extract of 9-day-old chick embryos in an equal volume of balanced salt solution.
of the embryos died on the 5th to 8th day post infection, and the skin of these embryos as well as internal organs, such as liver and kidneys, was extremely hemorrhagic. The longer the embryo lived, the less obvious and widespread were these hemorrhagic areas. Some strains had to be passed 3 or 4 times before the characteristic embryo mortality pattern was observed. The infectious particles were found in the allantoic-amniotic fluid as well as in the yolk, and the titer in each fluid was approximately the same. For instance, for the 10th

<table>
<thead>
<tr>
<th>TABLE I</th>
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<tbody>
<tr>
<td>A Comparison of the Mean Day of Deaths Obtained by Injecting 2 Strains of the Tendovaginitis Agent via the Yolk, Amniotic, Chorioallantoic Membrane, and Allantoic Route</td>
</tr>
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</table>

<table>
<thead>
<tr>
<th>Route of inoculation</th>
<th>Yolk</th>
<th>Amniotic</th>
<th>Chorioallantoic membrane</th>
<th>Allantoic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strain Lombardi (passage 9)</td>
<td>6.7</td>
<td>8.6</td>
<td>12.0</td>
<td>12.9</td>
</tr>
<tr>
<td>Strain 1071 (passage 3)</td>
<td>6.6</td>
<td>8.3</td>
<td>11.0</td>
<td>12.2</td>
</tr>
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</table>

<table>
<thead>
<tr>
<th>TABLE II</th>
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</thead>
<tbody>
<tr>
<td>The Effect of Various Dilutions of Antibiotics on the Survival Time of Embryos Infected with the 10th Passage of Strain 909</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Per cent protection (prolongation of mean day of death)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5000</td>
</tr>
<tr>
<td>Achromycin</td>
<td>ND*</td>
</tr>
<tr>
<td>Terramycin</td>
<td>ND</td>
</tr>
<tr>
<td>Aureomycin</td>
<td>ND</td>
</tr>
<tr>
<td>Chloromycetin</td>
<td>ND</td>
</tr>
<tr>
<td>Dihydrostreptomycin</td>
<td>83</td>
</tr>
<tr>
<td>Penicillin†</td>
<td>24</td>
</tr>
</tbody>
</table>

* ND, not done.
† Units per egg.

egg passage of strain 909, the LD$_{50}$ of the allantoic-amniotic fluid was $10^{-4.1}$ while the LD$_{50}$ of the yolk was $10^{-4.28}$.

Route of Inoculation.—Results of chick embryos inoculated by various routes showed the best route to be the yolk sac, followed by the amniotic sac, then the chorioallantoic membrane. The allantoic sac was markedly inferior to the others (Table I).

Filtration experiments indicated that the tendovaginitis agent will pass through a bacterial retaining filter, however, with a drop in titer. Allantoic-
TENDOVAGINITIS WITH ARTHRITIS

Amniotic fluid harvested from the 11th passage of strain 909 has a prefiltering LD₅₀ titer of 10⁻⁴.⁶ and a postfiltering titer of 10⁻³.⁶.

Antibiotic Sensitivity.—The results of antibiotic titrations with a pool of the yolk harvested from the 10th passage of strain 909 indicated that this agent is most sensitive to terramycin, a chromycin, perhaps slightly less sensitive to aureomycin, not as sensitive to dihydrostreptomycin and chloromycetin, and quite resistant to penicillin (Table II). Four other strains; 1071, 1464–29 (hock), 1464–29 (mucus), and Lombardi, when tested with 1 mg./egg of terramycin, dihydrostreptomycin, and 1000 units/egg penicillin showed the same antibiotic sensitivity pattern; i.e., most sensitive to the tetracyclines, not as sensitive to dihydrostreptomycin, and quite resistant to penicillin (Table III).

<table>
<thead>
<tr>
<th>Antibiotic per egg</th>
<th>Per cent protection (prolongation of the mean day of death)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Terramycin, 1 mg.</td>
<td>Dihydrostreptomycin, 1 mg.</td>
</tr>
<tr>
<td>Strain 1071 (passage 3)</td>
<td>98</td>
</tr>
<tr>
<td>Strain Lombardi (passage 7)</td>
<td>100</td>
</tr>
<tr>
<td>Strain 1464 mucus (passage 14)</td>
<td>90</td>
</tr>
<tr>
<td>Strain 1464 hock (passage 20)</td>
<td>100</td>
</tr>
</tbody>
</table>

Electron Microscopy of Strain 1071.—The supernatant and sediment resulting from the first low speed, high speed cycle of centrifugation (2000 r.p.m., then 25,000 G) had a LD₅₀ titer of approximately 10⁻¹.⁵ and 10⁻³.₈, respectively. This indicated that the centrifugal force used was sufficient for sedimenting the infectious particles. Electron micrographs of the crudely purified allantoic-amniotic fluid showed cocobacillary elements, from 0.2 to 0.5 μ in size, containing what appeared to be a limiting membrane that surrounded protoplasm of varying density (Fig. 1). No such elements were visible in the normal uninfected allantoic-amniotic controls.

Tissue Cultures.—Strain 909 repeatedly produced in roller tube tissue cultures of chicken heart fibroblast a cytopathogenic effect. Approximately 4 days after infecting, the fibroblasts became degenerative as exemplified by basophilic cytoplasm, pyknotic nuclei, and rounded-up cells. The uninfected controls and the heat-killed virus controls still appeared to have healthy, viable, fibroblasts (Fig. 2).

Host Range.—The white mice, pigs, and guinea pigs appeared in the gross to be unaffected by the injection of the tendovaginitis agent.
Organisms of the pleuropneumonia group have been known to be associated with arthritis in cattle, sheep, goats, pigs, rodents, and man (10-13). The peculiar characteristics of PPLO,—e.g. passage through a bacteria-retaining filter, scant growth on artificial media and difficult to detect, small size, tinctorial properties, and extreme resistance to penicillin,—make them easy to confuse with large viruses.

The above two facts; that PPLO can be readily confused with large viruses, and that PPLO have been associated with arthritis in a number of species, have impressed us with a need for great caution and reserve in trying to establish the nature of the etiological agent of this new arthritic disease of chickens. This caution and reserve were sharpened and focused when it was learned that the tendovaginitis agent had many of the characteristics that PPLO display in developing chick embryos. These common characteristics are antibiotic sensitivity spectrum, preference for yolk sac route, tinctorial properties, and size. These properties are also shared by rickettsiae and psittacoid viruses. It is for such reasons that many attempts were made to cultivate PPLO from the tendovaginitis agent.

The antibiotic sensitivity spectrum, the preference for the yolk sac route, the chick embryo mortality pattern, the results of filtration, suggest that the tendovaginitis agent can be either a large virus, rickettsia, or a PPLO. However, electron micrographs of the tendovaginitis agent show rigid, coccobacillary bodies that have limiting membranes surrounding protoplasmic areas of varying density. These structures seem distinct from those in electron micrographs of PPLO as published and reviewed by Morton et al. (14), these showing a lack of rigidity and density. The electron micrographs of the tendovaginitis agent on the other hand call to mind the published electron micrographs of rickettsiae (15).

Thus far PPLO isolated from cases of chronic respiratory disease of chickens and from non-specific urethritis of man have not produced in tissue cultures the cytopathogenic effect that results from infecting the same type of tissue culture with the tendovaginitis agent (16).

We have on record a case of a dual infection, chronic respiratory disease, and arthritis in the same bird. A PPLO was isolated from the trachea and readily grown on artificial media. This PPLO produced no joint lesions when injected into chickens. At the same time the tendovaginitis agent was isolated from the joint exudate and serially passed 7 times in eggs, but it would not grow on artificial media.

According to our present knowledge, it is felt that the tendovaginitis agent is distinct from PPLO, since the electron micrographs of the tendovaginitis agent are similar to those of rickettsiae and different from those of PPLO, the
tendovaginitis agent produces a cytopathogenic effect in tissue cultures that has not been observed to be produced by PPLO, and the tendovaginitis agent has never been cultivated on media that have supported the growth of PPLO. It is suggested that the characteristics of the tendovaginitis agent most resemble a rickettsia or possibly a large virus.

SUMMARY

The findings with an infectious agent isolated from cases of tendovaginitis or arthritis of chickens were as follows:—

Cultures, stains, and darkfield studies of material containing this agent for spirochetes, bacteria, and pleuropneumonia-like organisms were negative.

The yolk sac was the preferred route of inoculation, all of the embryos dying in 4 to 12 days.

The agent passed through a bacteria-retaining filter, but with a drop in titer.

Antibiotic sensitivity tests revealed that the agent was most sensitive to the tetracycline antibiotics, less sensitive to chloromycetin, dihydrostreptomycin, and resistant to penicillin.

From electron micrographs it was concluded that the agent is a rigid, dense, coccobacillus from 0.2 μ to 0.5 μ in size.

The agent produced a cytopathogenic effect in tissue cultures of chick heart fibroblasts.

The agent had no gross effect on suckling or newly weaned mice, pigs, or guinea pigs.

Reasons for suggesting that the agent is most similar to a rickettsia or possibly a large virus are discussed.

The authors wish to express their thanks to Dr. D. R. Coman, Department of Pathology, for the use of the electron microscope.

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EXPLANATION OF PLATE 54

Fig. 1. Electron micrographs of strain 1071. The preparation was from allantoic-amniotic fluid that had been sedimented and washed 4 times at 25,000 G. The formalin-fixed cells were examined with a Philips electron microscope at 80 kv. × 50,000.

Fig. 2. The effect of strain 909, 10th egg passage, on fibroblasts obtained from explants of 12-day-old embryonic chick heart muscle. The explants incubated 4 days were infected with a drop of allantoic-amniotic fluid, incubated 4 additional days, and then stained with May-Greenwald Giemsa stain. The specimen on the left received the same treatment except that the allantoic-amniotic fluid had been heated at 56°C. for 30 minutes. It served as a control. × 200.
Control Infected (Lecce et al.: Tendovaginitis with arthritis)