CHRONIC RESPIRATORY DISEASE: THERAPEUTIC EFFECT OF DEOXYCORTICOSTERONE AND TYLOSIN AND IMMUNIZATION AGAINST *ESCHERICHIA COLI*, A SECONDARY INVADER

Thesis for the Degree of M. S.
MICHIGAN STATE UNIVERSITY

John E. Lund

1964

ABSTRACT

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by John E. Lund

Chronic respiratory disease is a respiratory infection of chickens, characterized by tracheal rales, coughing, and lowered egg production or reduced growth rate. Mycoplasma gallisepticum, a pleuropneumonia-like organism (PPLO), is considered to be the causative organism. Gram-negative organisms, Escherichia coli in particular, are commonly encountered as secondary invaders.

The efficacy of treatment or prophylaxis of CRD with antibiotics or other therapeutic agents has been evaluated by a large number of investigators. In general, most of the therapeutic agents do not exert any marked inhibitory effect on the course or severity of the disease.

The purpose of this study was to evaluate the in vitro and in vivo response of M. gallisepticum to deoxycorticosterone (DOC) and tylosin both alone and in combination. The feasibility of immunizing chickens against the pathogenic effects of E. coli O11 was also investigated.

DOC in ethanol-chloroform and tylosin in ethanol were added to

sterile tubes and the solvent was evaporated. Grumble's medium with penicillin and thallium acetate was added to the tubes. An inoculum containing M. gallisepticum was added to each tube, and after incubation, the tubes were examined for the presence of PPLO. Uninoculated control tubes, solvent controls, and compound carry-over controls were prepared. DOC did not exhibit any inhibitory effect on the growth of M. gallisepticum. Tylosin at a concentration of 0.1 ug/ml of medium effectively inhibited the growth of the PPLO.

In the in vivo experiment, 1 ml of an egg yolk culture of M. gallisepticum was injected into the abdominal air sac of chickens. When the birds had begun to cough and show signs of respiratory distress, the birds were treated by the intramuscular injection of 25 mg of tylosin, or 20 mg of DOC, or with both drugs. When the infected birds, treated with tylosin, were compared with the infected non-treated birds, the number of isolable organisms decreased, weight gains increased, gross pathology decreased, and feed conversion increased in the treated birds. DOC had no observable effect as a therapeutic agent toward the air sac lesions and symptoms of experimentally produced CRD in chickens. The use of a combination of the two drugs resulted in no greater therapeutic response than that seen with tylosin alone.

Two methods were used to attempt to product anti-E. coli serum in chickens. The first procedure consisted of 6 subcutaneous injections of heat-treated E. coli at 2-day intervals. The second procedure consisted of 6 subcutaneous injections at 2-day intervals using a mixture of heat-treated E. coli organisms and specific lipopolysaccharide.

Passive immunization of the chickens against E. coli was attempted by the use of rabbit anti-E. coli serum. The rabbits were

immunized using the simultaneous injection of heat-treated E. coli and specific lipopolysaccharide. The injections were into the toe pads of the rabbits.

The active and passive immunization of chickens against the E. coli organism resulted, on challenge with live organisms, in protection against death but in no protection against the fibrinous inflammatory lesions involving the air sacs, liver capsule, and pericardium.

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A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Microbiology and Public Health

1964

ACKNOWLEDGEMENTS

I wish to express my sincere appreciation to Dr. Delbert E. Schoenhard for his personal and academic guidance and for the cordial friendship he has extended to me.

I would also like to thank my wife, Carol, for her help preparing this manuscript.

This study was supported by Ciba Pharmaceutical Company, Summit, New Jersey. This help was sincerely appreciated.

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INTRODUCTION

Chronic respiratory disease (CRD) is one of the most important disease entities now facing the poultry industry. It is a respiratory infection of chickens characterized by tracheal rales, coughing, and lowered egg production or reduced growth rate. The economic losses to the poultry industry from reduced egg production, inefficient feed conversion, and carcass condemnations at the market are above 25 million dollars annually. Respiratory lesions due to CRD and other causes are the largest single cause contributing to carcass condemnations. In 1960, 74 percent of carcass rejects were due to CRD (Rosenberg et al., 1962).

Chronic respiratory disease is caused by a pleuropneumonia-like organism, Mycoplasma gallisepticum. The disease was first described by Nelson (1935), indicating an etiological role for the pleuropneumonia-like organism, which he called cocco-bacilliform bodies. This work was later substantiated by Delaplane and Stuart (1943), who were the first to call this disease chronic respiratory disease. They described the propagation of the "virus" agent in embryonated chicken eggs. Markham and Wong (1952) first identified the pleuropneumonia-like organism as the etiological agent, later termed Mycoplasma gallisepticum by Edward and Kanarek (1960) to differentiate this organism from the non-pathogenic avian species of PPLO, Mycoplasma gallinarum.

The infection is transmitted by aerosol inhalation and by egg transmission[7]. Egg transmission is of a higher frequency in flocks that have had a recent occurrence of CRD than in flocks in which the disease

has become stabilized (Olesiuk and VanRoekel, 1960a).

Lecce and Sperling (1955) stressed the importance of secondary invaders in the initiation of the air sac lesions of chronic respiratory disease in chickens. Suggested as possible invaders in chronic respiratory infections are coliforms, Proteus, Micrococcus, and Aspergillus (Fahey and Crawley, 1955; Brandly, 1955). Price et al. (1957) described a shift from a predominantly gram-positive to a predominantly gram-negative flora with the occurrence of CRD.

Smibert et al. (1959) were able to produce all symptoms of CRD by inoculation of Mycoplasma gallinarum into germ-free chickens and turkeys. They were unable to explain the role of secondary invaders in CRD.

Beckman et al. (1959) divide the course of the disease into three stages. The first being an inapparent infection, with Mycoplasma gallisepticum present, but the birds not exhibiting any signs of infection. Stress initiates the second stage, when the symptoms of the disease are first apparent. Stress can come from a natural virus infection, vaccination for infectious bronchitis or Newcastle disease heavy laying or a sudden change in the weather. The third stage, when exudative lesions occur in the air sacs, is caused by the invasion of the air sacs by bacteria and/or fungi.

Gross (1956) reported a disease characterized by a fibrinous perihepatitis, pericarditis and airsacculitis which had been observed as a complication of CRD of chickens. Wasserman et al. (1954) reported on 22 birds characterized by the above lesions, from which Escherichia coli was isolated in all cases. Gross (loc. cit.) studied the possibility of vaccinating chickens against E. coli. He used a formalin-killed organism,

an "O" antigen vaccine obtained by heating the organism at 100°C for one hour, and live-organism vaccines given intravenously, intramuscularly, intraperitoneally and as an aerosol spray. The killed vaccines produced a high "O" antigen titer but offered no more resistance to challenge than that seen in normal birds. All of the live-organism vaccines gave good protection but tended to produce severe reactions following vaccination. Only the aerosol vaccination method gave good protection without producing severe reactions in the birds, however, when Mycoplasma gallisepticum was present in the vaccinated birds, losses often occurred. Gross also observed that there did not appear to be any correlation between the "O" antibody titer and immunity following exposure to killed organisms but the "O" antibody titer was a good indication of immunity following the exposure to live organisms.

The efficacy of treatment or prophylaxis of CRD with antibiotics or other chemotherapeutic agents has been evaluated by a large number of investigators. Forty different agents, and/or combinations have been investigated (see Table 1). As indicated in Table 1, the results of these investigations are at times contradictory. Some of the reasons suggested for the variations in the results of these investigations are: the severity of the infection, strain differences in the bacteria and chicken, the number and kind of secondary invaders, the kind and amount of stress to which the birds are subjected, variations in housing, light, and nutrition, and differences in methodology and interpretation of results (Padgett, 1961).

The criteria used to evaluate the efficacy of a therapeutic agent are: a decrease in the gross pathological changes in the air sacs, improved weight gains over untreated controls, and an increase in the

Table 1. Antibiotics and chemotherapeutic agents which have been used against CRD in chickens

Agent	Not Effective	Moderately Effective	Effective
Aureomycin	19*,5,6,7	10,11,12, 13,9	20,3,21
Aureomycin + Terephthalic acid		12,13	
Bicillen	11		
Bicillen + Streptomycin	4		
Chloramphenicol	8,19		
Deoxycorticosterone			14
6-Demethylchlortetracycline		21	
Dihydrostreptomycin	11		
Dihydrostreptomycin + Propylene glycol			15
Erythromycin	7	2	
Erythrocin	11		
Furaltadone		7	
Furazoldone		11	
Leukomycin	9		
Magnamycin		11	
Ninhydrazone			18
Nitrofurazone + Furazolidone			18
Oxytetracycline	19,5,6	10,11,16, 12,2	3,17,21
Pabakay	11		
Penicillin	3,16,17		
Penicillin + Streptomycin	4		5

Table 1 (continued)

Agent	Not Effective	Moderately Effective	Effective
Polyotic	11		
Spiramycin	11		
Streptomycin	5,4,11	5,9	
Streptomycin + Dihydrostreptomycin		2	
Terramycin + Terephthalic acid		12	
Tetracycline		9	21
Tylosin		1	

*Refers to reference in the Selected Bibliography.

Selected Bibliography

1. Barnes, L. E., E. E. Ose, and F. O. Gossett, 1960.
2. Blake, J. T., 1961.
3. Boone, M. A., D. J. Richey, and C. L. Morgan, 1957.
4. Cover, M. S., 1955.
5. Fahey, J. E., and J. F. Crawley, 1954.
6. Fahey, J. E., and J. F. Crawley, 1955.
7. Gross, W. B., 1961.
8. Heishman, J. O., N. O. Olson, and C. J. Cunningham, 1962.
9. Kiser, J. S. F. Popken, and J. Clemente, 1960.
10. Lecce, J. G., and F. G. Sperling, 1955.
11. Olesiuk, O. M., H. VanRoekel, and L. P. Beninato. 1957.
12. Olesiuk, O. M., and H. VanRoekel, 1959.
13. Olson, N. O., D. C. Shelton, and J. O. Heishman, 1959.

Table 1. Selected Bibliography (continued)

14. Padgett, G. A., and D. E. Schoenhard, 1959.

15. Perek, M., and K. Perek, 1958.

16. Price, K. E., Z. Zoli, Jr., W. B. Hardie, and M. J. Gallian, 1957.

17. Price, K. E., Z. Zoli, Jr., W. B. Hardie, and R. Owens, 1958.

18. Rosenberg, D. V., W. D. Woodward, and A. E. Kline, 1962.

19. VanRoekel, H., O. M. Olesiuk, and H. A. Peck, 1952.

20. White-Stevens, R., and H. G. Zeible, 1954.

21. Whitehill, A. R., and J. S. Kiser, 1961.

feed conversion ratio over untreated controls. Some of the therapeutic agents stimulated an increase in the weight gain of infected birds above that obtained in untreated controls, while others did not (Olesiuk and VanRoekel, 1959; Heishman et al., 1962; Gross, 1961; Price et al., 1958). One agent, furaltadone, depressed growth below that observed in untreated controls (Gross, 1961). As an example of an agent reducing the gross pathological lesions in the air sacs, feed containing nitrofurazone and furazolidone fed to chickens, resulted in a significant reduction (3.5 percent versus 27.5 percent) in the total number of condemned carcasses at slaughter (Rosenberg et al., 1962).

In general, most of the therapeutic agents do not exert any marked inhibitory effect on the course or severity of the disease.

Padgett and Schoenhard (1959) reported they had obtained excellent results in the treatment of CRD using an adrenocorticosteroid, deoxycorticosterone (DOC). When birds treated with DOC were compared with untreated controls, the number of isolable organisms decreased, weight gains increased, gross pathology decreased and feed conversion improved in the treated birds.

This thesis will report on the in vitro and in vivo response of Mycoplasma gallisepticum, the causative organism of CRD, to deoxycorticosterone and tylosin, both alone and in combination. The feasibility of immunizing chickens against the pathogenic effects of Escherichia coli O11 was also investigated.

This study was divided into four parts:

Experiment I: The in vitro activity of DOC and tylosin against Mycoplasma gallisepticum.

Experiment II: The in vivo response of Mycoplasma gallisepticum

to DOC and tylosin.

Experiment III: Active immunization of chickens against *E. coli* O11.

Experiment IV: Passive immunization of chickens against *E. coli* O11 using rabbit anti-*E. coli* serum.

MATERIALS AND METHODS

Experiment I: The in vitro activity of DOC and tylosin against M. gallisepticum. The culture of M. gallisepticum used in this experiment and Experiments II and IV was obtained from Dr. Olga M. Olesiuk, Paige Laboratories, University of Massachusetts. The organism was an S-6 strain. It was maintained and stored frozen in egg yolk.

DOC in ethanol-chloroform, 1:1, was added to sterile tubes and the solvent was evaporated. Tylosin in ethanol was added to sterile tubes and the ethanol evaporated (see Table 3). Grumble's medium with penicillin, 2000 units per ml, and thallium acetate, 1 gm per 2000 ml (Taylor and Fabricant, 1957) was added to the tubes. An inoculum containing approximately 1×10^6 M. gallisepticum was added to each tube. To be certain that the solvents used were not inhibitory to the growth of the PPLO, tubes were prepared with the solvents, the solvents evaporated, and medium and PPLO inoculum added. Control tubes were also prepared with just the medium and PPLO inoculum. Uninoculated control tubes were prepared, in which DOC and tylosin were added to the medium. The tubes were incubated at $37^{\circ}C$ for a period of 48 hours beyond the time that the last medium control tube changed color.

An observation of a color change in the medium indicated the growth of the PPLO. To confirm this, samples were cultured on PPLO agar (Difco) with thallium acetate, 1 gm per 2000 ml and enriched with 2 percent serum fraction (Difco). Likewise samples were taken from all tubes in which no color change was noted. To ensure that the absence of

PPLO growth on the agar was not due to the carry-over of tylosin or DOC from the tubes, medium from the color control tubes was spotted on PPLO agar and after drying, PPLO was added to the test medium spots. The plates were incubated at 37°C for 72 hours, in a closed jar with enough water present to provide a humid atmosphere. The presence of PPLO colonies was checked at 100X magnification.

Experiment II: The in vivo response of M. gallisepticum to DOC and tylosin. The culture of M. gallisepticum used in this experiment was obtained from Dr. Olga M. Olesiuk, University of Massachusetts. The organisms were cultivated in the yolk sac of 7-day old chicken embryos. The yolk material was harvested after 5 days of incubation.

A total of 93 apparently disease-free, White Leghorn cockerels were utilized in this experiment. The birds were obtained when they were one day old from the Rainbow Trails Hatchery, St. Louis, Michigan, and were raised in conventional brooders. When the birds were 4 weeks old, they were randomly divided and placed in 5 isolation pens. The chickens were fed a commercial, antibiotic-free grower ration. Feed consumption was measured at the beginning of the experiment when the birds were 6 weeks old. All birds were weighed at the beginning of the experiment, and when they were removed from the pens.

Seventy-two birds (18 in each of Pens 2, 3, 4, and 5) were inoculated with M. gallisepticum and 21 birds (Pen 1) were maintained to serve as uninoculated controls. The birds were inoculated by injecting 1 ml of M. gallisepticum inoculum, containing approximately 5×10^5 organisms per ml, into the abdominal air sac. The injection was made through the body wall, at the level of the last rib, using a 5 cc syringe with a 20 gauge, 1½ inch needle. The inoculum consisted of equal portions

of PPLO broth (Difco) with 2 percent serum fraction (Difco) and 4,000 units of penicillin per ml and yolk material obtained from 10, twelve-day old embryonated chicken eggs which had been inoculated at 7 days of age with 0.1 ml of egg yolk containing approximately 1 x 10^5 organisms. The experimental design is shown in Table 2.

When the experimental birds had begun to cough and show signs of respiratory distress, a random sample was removed from each pen, weighed and killed. The birds were examined for gross pathological lesions of CRD and an attempt was made to enumerate the M. gallisepticum bacteria from the thoracic air sacs. Portions of the air sacs were excised, weighed and placed in 1 ml of PPLO broth (Difco) enriched with 1 percent serum fraction (Difco). The air sac material was ground and 4 tenfold dilutions were made. One-tenth ml of each dilution was spotted on PPLO agar (Difco) enriched with 4 percent serum fraction (Difco) and containing a 1:2000 concentration of thallium acetate. The agar plates were incubated at 37°C for 72 hours in a closed jar, containing enough water to provide a humid atmosphere. The number of organisms per 0.1 ml drop was counted at 100X magnification and the number of organisms per mg of tissue was calculated.

Treatment of the infected birds was instituted on the day respiratory distress was first observed. Tylosin was injected into the breast muscle at the level of 25 mg per bird. DOC was injected intramuscularly at the level of 20 mg per bird.

At 7 and 14 days post-treatment a sample of the birds was removed, weighed, and killed. The birds were examined for gross pathological lesions of CRD and attempts were made to enumerate the M. gallisepticum organisms from the thoracic air sacs. Of the birds remaining,

Table 2. Design of Experiment II.

Pen		Number of birds	Treatment	Days post-infection 7	14	21	28
1.	non-infected	21	none	kill 5 and culture	kill 4 and culture	kill 6 and culture	kill 6 and culture
2.	infected	18	none	kill 2 and culture	kill 4 and culture	kill 6 and culture	kill 6 and culture
3.	infected	18	tylosin, 25 mg im, 7, 14, 21 days post-infection.	treat 16, kill 2 and culture	treat 12, kill 4 and culture	treat 6, kill 6 and culture	kill 6 and culture
4.	infected	18	DOC. 20 mg im, 7 days post-infection.	treat 16, kill 2 and culture	kill 4 and culture	kill 6 and culture	kill 6 and culture
5.	infected	18	tylosin, 25 mg im, 7, 14, 21 days post-infection. 20 mg DOC im. 7 days post-infection	treat 16, kill 2 and culture	treat 12, kill 4 and culture	treat 6, kill 6 and culture	kill 6 and culture

those which had received tylosin on the first treatment day were injected with 25 mg of tylosin per bird.

On the twenty-first day post-treatment, the remaining birds were killed, weighed, and examined for gross pathological lesions of CRD and attempts were made to enumerate the M. gallisepticum organisms from the thoracic air sacs.

Experiment III: The active immunization of chickens against E. coli O11. The culture of E. coli used in this experiment was obtained from Dr. W. B. Gross, Virginia Polytechnic Institute. The organism was designated O11. It was isolated from a chicken which had CRD with pericarditis and perihepatitis and was considered pathogenic for the chicken. The organism was maintained as a nutrient agar stab culture.

Two methods were used to attempt to produce anti-E. coli serum in chickens. One method consisted of the subcutaneous injection of heat-treated E. coli organisms. The second method utilized the simultaneous injection of heat-treated E. coli organisms and the lipopolysaccharide extracted from the E. coli cell wall (Seigneurin et al., 1962).

The lipopolysaccharide was extracted from the cell wall by the phenol-water method (Kabet and Mayer, 1961). The E. coli culture was grown for 18 hours in broth containing tryptose (Difco), NaCl, thiamine HCl, and dextrose at 20 gm, 5 gm, 0.56 mg and 1 gm respectively, per liter of distilled water, and harvested by centrifugation. The crude product obtained by the phenol-water extraction contained a large amount of nucleic acids and was fractionated by repeated ethanol precipitation and was further purified by centrifugation at 95,000 RCF for one hour.

To gain some knowledge of the composition of the lipopolysaccharide, the following assays were performed: protein content, total

carbohydrate, ribonucleic acid (RNA) content, and deoxyribonucleic acid (DNA) content. The 50 percent lethal dose (LD_{50}) of the endotoxin for mice was also determined.

The carbohydrate content was determined as anthrone positive substances (Morris, 1948). The protein content was determined using the method of Folin-Ciocalteu (Cowgill and Pardee, 1957) with bovine serum albumin as the standard. The DNA and RNA content was also evaluated by the use of colorimetric methods. Diphenylamine-acetaldehyde reagent was used for the DNA determination and orcinol reagent for the RNA determination (Chargaff and Davidson, 1955). The LD_{50} of the lipopolysaccharide for mice was determined by intraperitoneal injection. Five White Swiss mice, average weight 16.2 gm, were used for each of the following quantities: 12.5, 25, 50, 100, 200, and 400 ug. The LD_{50} was calculated by the statistical method of Reed and Muench (1938).

The first immunization procedure consisted of 6 subcutaneous injections of heat-treated E. coli, using 2.5×10^6 organisms per bird, at 2-day intervals. Four White Leghorn chickens approximately 4 months old were used. The E. coli organisms were grown in tryptose broth containing NaCl, thiamine HCl, and dextrose, at 37°C. The organisms were harvested, washed with 0.85 percent saline solution and were heated at 56°C for 1 hour.

The second immunization procedure consisted of 6 subcutaneous injections at 2-day intervals, using a mixture of 2.5×10^6 E. coli organisms from an 18-hour broth culture, heated at 56°C for 1 hour and 10 ug of lipopolysaccharide per bird. The lipopolysaccharide had been treated with 0.25 N NaOH for one hour at 56°C and adsorbed on a 5 percent suspension of rabbit erythrocytes at 37°C for one hour. Prior to the

adsorption of the lipopolysaccharide, the erythrocytes were stored in Alsever's solution. A total of 12 Leghorn chickens were used. Eight of them were four months old and 4 of them were one month old.

Fifteen days after the final immunizing injection, a blood sample was taken from a random sample of both groups. The serum was inactivated by heating at 56°C for ½ hour. The serum titer for the "O" and "H" antigens was determined by the tube agglutination method. The serum was initially diluted 1:4 with 0.85 percent saline solution and seven two-fold dilutions were made so that 0.5 ml of each dilution remained in each tube. One-half ml of "O" and "H" antigen preparation was added, each to its own row of tubes. The tubes were incubated at 52°C for 4 hours and stored at 4°C for 12 hours before the agglutination titer was determined.

The "H" antigen was prepared by suspending a thrice-washed 18-hour culture of E. coli, grown on BHI agar, in 0.85 percent NaCl with 0.2 percent formalin. The density corresponded to a McFarland nephlometer tube number 6.

The "O" antigen was prepared by making a thick suspension of a washed E. coli culture and adding an equal volume of ethyl alcohol, while stirring. The preparation was covered and stored for 24 hours at 37°C. The bacterial cells were resuspended and an equal volume of 0.85 percent saline was added. Before each agglutination test, a portion of this stock suspension was diluted with 0.85 percent saline to obtain a density corresponding to a McFarland nephlometer tube number 6.

After the blood samples were obtained, on the fifteenth day, both groups of immunized birds and 6 control birds were challenged by injecting into the abdominal air sac 3.8×10^5 E. coli organisms

harvested from BHI agar plates and washed with 0.85 percent NaCl.

Because the serum titers were low before the challenge, blood samples were obtained from the birds at 4-day intervals until a period of 16 days had passed. The serum was frozen until the last sample was obtained at which time all samples were inactivated at 56°C for ½ hour, and the agglutination titer for both the "O" and "H" antigens was determined.

Twenty days after the challenge, the birds were killed and examined for gross pathological lesions.

Experiment IV: The passive immunization of chickens against E. coli O11 using rabbit anti-E. coli serum. The culture of E. coli used in this experiment was strain O11, which was obtained from Dr. W. B. Gross, Virginia Polytechnic Institute.

Five mature Dutch Belt rabbits were used for the production of anti-E. coli serum. The immunization procedure consisted of 6 two-tenths ml injections at 2-day intervals, using an inoculum of 2.5×10^6 E. coli and 10 ug of lipopolysaccharide adsorbed on rabbit erythrocytes. The culture of E. coli was grown on BHI agar and was heated at 56°C for one hour. The lipopolysaccharide had been treated with 0.25 N NaOH for 1 hour at 56°C and was adsorbed on rabbit erythrocytes at 37°C for 1 hour. The injections were given in the toe pads of the rabbits, while they were anesthetized with ether.

Fifteen days after the final injection, blood samples were drawn from the ear vein of each of the rabbits and after the serum was inactivated, the tube agglutination titers for the "O" and "H" antigens were determined. The antigens were prepared by the same methods as outlined in Experiment III. The agglutination titers were determined

after 4 hours at 56°C and 12-24 hours at 4°C. This procedure was followed at all times during this experiment.

On the eighteenth and twenty-fifth day after the final immunization injection, booster injections were given to each of the rabbits. The booster injections consisted of 1×10^7 heat-treated E. coli organisms and 10 ug of lipopolysaccharide adsorbed on homologous erythrocytes. The vaccine was injected into the toe pads while the rabbits were under ether anesthesia.

Seven and 14 days after the second booster injection, blood samples were collected from the ear vein of each of the rabbits. The tube agglutination titer for the "H" and "O" antigens was determined.

On the twenty-first and twenty-eighth days following the second booster injection, approximately 20 ml of blood were collected from each of the rabbits by means of a cardiac puncture, using a 20 cc syringe with a 20 gauge, 1½ inch needle. The "O" and "H" antigen agglutination titers were determined for the serum of each of the rabbits before the serum was pooled.

Thirty-five days after the second booster, a third booster injection was given to each rabbit. The material for the third booster was the same as the first and second.

Sixteen days following the third booster injection, approximately 20 ml of blood were drawn from each rabbit by means of cardiac puncture. The agglutination titer for the "H" and "O" antigens was determined.

A portion of the pooled serum was fractionated by $(NH_4)_2SO_4$ precipitation to separate the gamma fraction from the other serum proteins. All work done with the serum was carried out at 4°C. To one volume of cold serum one volume of distilled water was added; while the

serum was being stirred, two volumes of 70 percent saturated $(NH_4)_2SO_4$ were added, drop by drop with a separatory funnel. The precipitate was allowed to settle for ½ hour and was separated from the supernatant fluid by centrifugation. The precipitate was resuspended in distilled water to the original volume and was reprecipitated using an equal volume of 70 percent saturated $(NH_4)_2SO_4$. The precipitate was recovered by centrifugation and suspended in distilled water to 4/5 of the final desired volume. The serum gamma fraction was then dialyzed against 0.85 percent NaCl for 72 hours, with frequent changes of the dialysis fluid. After dialysis, 0.85 percent NaCl was added to attain a final volume of ½ the original volume.

In an attempt to find the minimal dose of serum which would protect chickens against the pathological effects of the E. coli bacteria and to determine how long the serum remained effective, thirty-two 6-week old White Leghorn chickens were injected intraperitoneally with 0, 0.5, 1, and 1.5 ml of the gamma fraction of the anti-E. coli serum. On the second, fourth, sixth, and eighth days following the serum injection, 2 birds from each group were challenged with an intraperitoneal injection of 1×10^5 E. coli organisms, harvested from BHI agar plates, incubated for 18 hours. The birds were examined for gross pathological lesions either at the time of death or when 1 week had passed since the challenge.

To determine if there was a difference in the protection afforded by the use of the gamma fraction or whole immune serum, a total of 22 six-week old Leghorn chickens were injected with either 4 ml of whole anti-E. coli serum or 2 ml of gamma fraction of the anti-E. coli serum. On the second and fourth days after the serum was administered by intraperitoneal injection, 4 birds from each group and 4 control birds

were given intraperitoneal injections of 5×10^4 *E. coli* organisms prepared from an 18-hour BHI agar plate culture. On the sixth day, 3 birds from each group and 3 controls were similarly challenged. The birds were killed and examined for gross pathological lesions when one week had passed since the live-organism challenge.

RESULTS

Experiment I. DOC did not exhibit any inhibitory effect on the growth of M. gallisepticum in Grumble's medium, even at the level of 1 mg per ml.

Tylosin effectively inhibited the growth of M. gallisepticum in vitro. Growth was inhibited by as low as 0.01 ug per ml of tylosin although at this level it was not bacteriocidal.

As shown in Table 3, the solvents used for DOC and tylosin did not exhibit any effect on the growth of M. gallisepticum in Grumble's medium.

Experiment II. Tracheal rales and coughing were noted in the infected birds on the seventh day following inoculation with M. gallisepticum. At this time 5 birds were removed from Pen 1 and 2 birds were removed from each of Pens 2, 3, 4, and 5. Treatment was instituted in the remaining birds of Pens 3, 4, and 5. All of the infected birds exhibited lesions which were typical of early CRD. The air sacs were cloudy and heavy with moist exudate. The air sacs from the non-infected control birds were clear and the birds appeared to be in good condition.

On the seventh day post-treatment, 4 birds from each pen were removed, weighed, and killed. The necropsy examination indicated the non-infected control birds from Pen 1 were normal. The birds from Pen 2, the infected, non-treated controls, had severe air sac lesions, typical of CRD. Caseous plaques were present on the thoracic and abdominal air sacs. Frequent coughing was noted in the birds in the pen

Table 3. The *in vitro* activity of DOC and tylosin against *M. gallisepticum*

Compound	Tube Number					
	1	2	3	4	5	6
Deoxycorticosterone						
level of DOC	1.0 mg*	0.5 mg	0.25 mg	0.125 mg	0.0625 mg	0.032 mg
PPLO growth	yes	yes	yes	yes	yes	yes
PPLO recovered	yes	yes	yes	yes	yes	yes
color change, control tube	no	no	no	no	no	no
carry over inhibition on PPLO agar	no	no	no	no	no	no
Tylosin						
level of tylosin	1000 ug*	100 ug	10 ug	1 ug	0.1 ug	0.01 ug
PPLO growth	no	no	no	no	no	no
PPLO recovered	no	no	no	no	no	yes
color change, control tube	no	no	no	no	no	no
carry over inhibition on PPLO agar	yes	yes	yes	no	no	no
PPLO Grumble's medium control, growth	yes	yes	yes	yes	yes	yes
Ethanol control, growth	yes	yes	yes	yes	yes	yes
Ethanol-chloroform control, growth	yes	yes	yes	yes	yes	yes

*per ml, 5 ml total volume

prior to their removal. The birds in Pen 3, treated with tylosin, had ceased to cough and it was observed upon necropsy examination of the 4 birds removed that the air sacs were cloudy but the moist exudate was greatly diminished; some lymphofollicular "beading" (Olesiuk and VanRoekel, 1960b) was present. The birds in Pen 4 coughed frequently and moist rales were noted when the birds became excited. The gross pathological lesions were similar to those seen in Pen 2. Slight coughing was noted in the birds of Pen 5 which were treated with DOC and tylosin. Necropsy examination of 4 birds revealed 2 of them had severe lesions, typical of CRD, while 2 birds had comparatively mild lesions. The air sacs were cloudy, and a minimal amount of moist exudate was present.

Examination of the birds 14 days following treatment revealed coughing and moist rales only in one pen, Pen 4, the birds which had received DOC. Six birds were removed from each of the pens. They were weighed, killed and examined for gross pathological lesions of CRD. The noninfected control birds, Pen 1, were normal, and in good condition. Four birds in Pen 2 exhibited 3+ pathological lesions (air sacs cloudy, moist and caseous plaques) while 2 exhibited 2+ lesions (opaque and dry). Of the six birds in Pen 3, two exhibited 4+ lesions (air sacs cloudy, very moist and numerous caseous plaques), three 1+ lesions (lymphofollicular beading) and 1 had clear air sacs. The birds in Pen 4 appeared to be in poorer condition than the infected non-treated controls. Five of 6 birds exhibited 4+ pathological lesions, while the remaining bird exhibited 1+ lesions. Three birds in Pen 5 contained no lesions of CRD, two had 3+ lesions, and one contained 1+ lesions.

On the twenty-first day post-treatment, examination of the air sacs of the remaining birds revealed: Pen 1, all normal; Pen 2, one 4+,

Table 4. The gross air sac pathology and average number of *M. gallisepticum* per mg of air sac tissue

	Days post-inoculation			
	7	14	21	28
Gross Pathology*				
Pen 1	0 (5)**	0 (4)	0 (6)	0 (6)
Pen 2	3+ (2)	4+ (4)	2+ (2), 3+ (4)	2+ (1), 3+ (3) 4+ (1)
Pen 3	3+ (2)	1+ (2), 2+ (2)	0 (1), 1+ (3) 4+ (2)	0 (3), 2+ (2) 3+ (1)
Pen 4	3+ (2)	4+ (4)	1+ (1), 4+ (5)	0 (1), 2+(1) 3+ (2), 4+ (2)
Pen 5	3+ (2)	2+ (2), 4+ (2)	0 (3), 1+ (1) 3+ (2)	0 (2), 2+ (4)
Organisms recovered				
Pen 1	0***	0	0	0
Pen 2	6.65×10^3	2.49×10^4	1.52×10^3	3.58×10^2
Pen 3	1.14×10^4	2.0×10^3	1.6	2.5
Pen 4	3.95×10^5	6.53×10^3	4.14×10^2	1.2×10^3
Pen 5	6.0×10^4	1.0×10^2	0	1.65×10^1

* 0 = no visible lesions, 1+ = lymphofollicular beading, 2+ = air sacs opaque and dry, 3+ = air sacs cloudy, moist, with some caseous plaques, 4+ = air sacs cloudy, very moist, with numerous caseous plaques.

** () = number of birds.

*** Organisms per mg of air sac tissue.

Table 5. Average weight gains and feed conversion

Pen number and description	Total food consumed in pounds	Total weight gain in pounds	Feed per pound of gain, in pounds	Percent increase in feed per pound gain over non-infected control birds
1. Noninfected, nontreated	63.5	16.5	3.85	---
2. Infected, nontreated	53.5	12.5	4.28	11.2%
3. Infected, treated with tylosin	55.0	13.5	4.07	5.7%
4. Infected, treated with DOC	54.5	12.5	4.36	13.2%
5. Infected, treated with tylosin and DOC	59.0	13.5	4.37	13.5%

three 3+, and one 2+; Pen 3, one 3+, two 2+, and 3 normal; Pen 4, two 4+, two 3+, and one 2+, and 1 normal; Pen 5, four 2+ and 2 normal. The gross pathological lesions noted in Experiment II are summarized in Table 4.

The weight gain and feed conversion figures for each pen are shown in Table 5.

The results of attempts to enumerate PPLO from the birds, at the time of treatment and after treatment are shown in Table 4. PPLO were not found in any of the non-infected controls.

Experiment III. On the basis of the Folin-Ciocalteu protein assay an average of 384 ug of protein per ml, or 7.7 percent of the lipopolysaccharide, was found.

The total carbohydrate, determined as anthrone positive substances, amounted to 1900 ug per ml, or 38 percent of the lipopolysaccharide.

The diphenylamine-acetaldehyde test for DNA was negative. The orcinol colorimetric determination for RNA was positive and 805 ug/ml or 16.1 percent of the lipopolysaccharide was calculated from a standard curve. Since the orcinol reaction has a low specificity, reacting with pentoses as well as 2-deoxyribose, DNA, methylpentose and hexuronic acids which absorb at 670 mu and certain aldoheptoses, which can occur in bacterial polysaccharides, and absorb at 655 mu (Chargaff and Davidson, 1955), a continuous absorption spectrum from 240 to 310 mu was obtained on 1:10 and 1:20 dilutions of the lipopolysaccharide and a standard containing 80 ug of RNA. The results of the absorption spectra are shown in Figure 1.

The results of the LD_{50} determination are presented in Table 6.

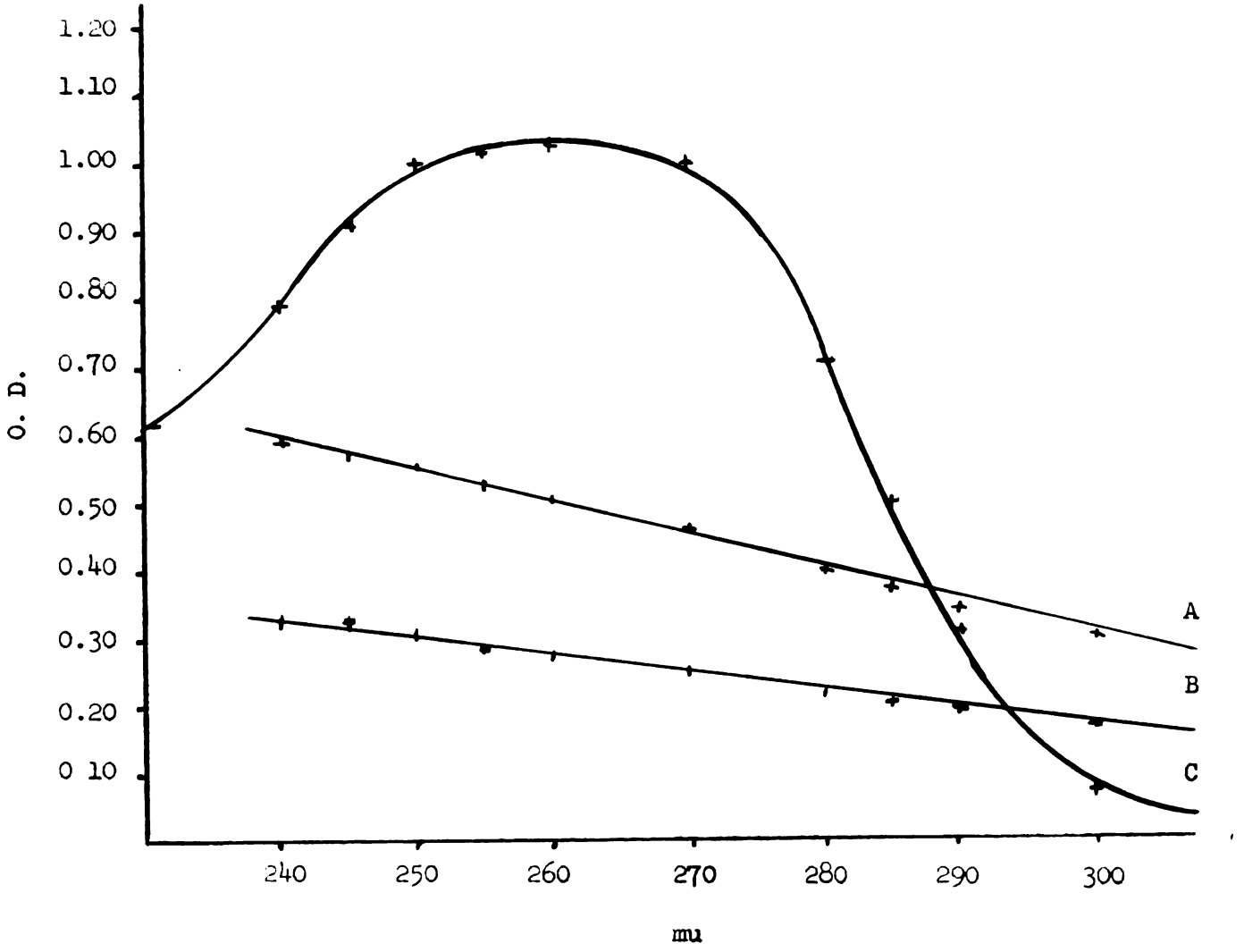

Figure 1. Absorption spectra of RNA standard and E. coli lipopolysaccharide. Symbols: A, one to ten dilution of lipopolysaccharide; B, one to twenty dilution of lipopolysaccharide; C, RNA standard, 80 ug per ml.

Table 6. Results of 50 percent endpoint of endotoxin in mice

		50 ug	100 ug	200 ug	400 ug
Mortality Rate		0/5	1/5	3/5	5/5
Died - Number		0	1	3	5
Survived - Number		5	4	2	0
Accumulation Totals	Died	0	1	4	9
	Survived	11	6	2	0
	Mortality Rate	0/11	1/7	4/6	9/9
	Percent Mortality	0	14.3	66.7	100

The LD_{50} was calculated using the method of Reed and Muench (1938). The LD_{50} was calculated to be 172.8 ug lipopolysaccharide per mouse, or 10.7 ug lipopolysaccharide/gm mouse.

The serum agglutination titers were low for the "H" and "O" antigens of the chickens 15 days after immunization with a culture of heat-treated E. coli or E. coli bacteria with lipopolysaccharide. The highest titer obtained was 32 for the "H" antigen and 16 for the "O" antigen. No difference in immune response was noted between the two groups of chickens.

All of the immunized birds survived the intraperitoneal injection of live E. coli organisms. All of the nonimmunized control birds died within 36 hours after the injection. Severe pericarditis, perihepatitis and airsacculitis were observed in the dead control birds.

The serum agglutination titer for the E. coli antigens remained low in all of the immunized birds after the challenge. The highest titers were 128 and 16 for "H" and "O" antigens, respectively, in four birds 16 days after the challenge.

Necropsy examination of the birds 20 days after the challenge disclosed pathologic lesions in all of the birds. The air sacs were cloudy and moist, and fibrinous inflammatory changes were present on the pericardium and liver capsule.

Experiment IV. The serum agglutination titers of the rabbits for the E. coli antigens are shown in Table 7.

As noted in Table 7, rabbit 339 died after the first booster injection. Just prior to the death of the rabbit, a profuse, hemorrhagic, discharge was observed flowing from the vulva. The skin and mucous membranes of the rabbit were blanched. Necropsy examination revealed that the rabbit had been bred during the immunization series, 20-25 days previous to death. Numerous petechial hemorrhages were present on the kidneys and a hemorrhagic placentitis was evident. The uterine lesions were similar to those described by Roberts (1949), who reported that one-tenth of the lethal dose of E. coli endotoxin would induce hemorrhages in the placenta and cause abortion in mice 15-18 days pregnant.

The E. coli "H" and "O" antigen agglutination titers for the pooled rabbit serum were both 2560. The gamma fraction titer ($\frac{1}{2}$ original volume) was 5120 for the "H" antigen and 2560 for the "O" antigen.

The attempt to immunize chickens against the pathological effects of E. coli was not successful, although some protection against death was afforded by the anti-E. coli serum (Table 8). Necropsy examination disclosed that the survivors had not been protected against the other effects of the organism. A majority of the survivors had airsacculitis, and severe fibrinous pericarditis and perihepatitis.

Table 7. Rabbit serum agglutination titers

Days after immunization*	15	26	33	40	47	54	80
Rabbit number							
328	O-1024 H-256	O-4096 H-256	O-1024 H-512	O-4096 H-2048	O-2048 H-2048	O-1024 H-1024	O-2048 H-4096
329	O-512 H-256	O-4096 H- -	O-2048 H-1024	O-2048 H-2048	O-2048 H-1024	O-1024 H-2048	O-2048 H-4096
331	O-256 H-512	O-4096 H-512	O-2048 H-512	O-2048 H-2048	O-1024 H-1024	O-1024 H-128	O-1024 H-2048
338	O-1024 H-512	O-4096 H-512	O-2048 H-512	O-4096 H-2048	O-2048 H-1024	O- - H- -	O-4096 H-4096
339	O-32 H-512	dead					

*Booster injections were administered at the nineteenth and sixty-first days.

Table 8. Challenge with E. coli of chickens passively immunized with rabbit anti-E. coli gamma fraction

Amount of gamma fraction	Number died over total				Birds with pathological lesions over total			
	2*	4	6	8	2	4	6	8
0	1/2	1/2	1/2	2/2	2/2	2/2	2/2	2/2
0.5 ml	1/2	2/2	1/2	-	2/2	2/2	1/2	-
1.0 ml	0/2	0/2	0/2	2/2	2/2	1/2	1/2	2/2
1.5 ml	0/2	2/2	0/2	2/2	2/2	2/2	1/2	2/2

* day challenged after serum gamma injection

A comparison of the use of whole or fractionated rabbit anti-E. coli serum to protect chickens revealed that both were equally non-effective. The number of organisms used to challenge the birds was not great enough to cause death in any of the birds, including the controls. All of the birds (11 injected with whole serum, 11 with fractionated serum and 11 normal controls) looked unthrifty, ruffled feathers and respiratory distress, within 18 hours after the intraperitoneal injection of the E. coli O11 pathogen. All birds, when killed one week after the challenge, had gross pathological lesions in the body cavity. The conditions seen were airsacculitis, pericarditis, and perihepatitis. There was no significant variation in the severity of the lesions among the 3 groups.

DISCUSSION

Deoxycorticosterone, an adrenal mineralocorticoid hormone, has been shown to exhibit antimicrobial activity against Saccharomyces fragilis (Maxwell et al., 1960), Trichophyton rubrum (Chattaway et al., 1963), Mycoplasma gallisepticum (Padgett and Schoenhard, 1959), and gram-positive bacteria (Lester and Hechter, 1958).

The M. gallisepticum organism, in contrast to other genera of bacteria (Tourtellotte, et al., 1963) and saprophytic PPLO (Lynn and Smith, 1960) contains cholesterol and cholesterol esters in the cell membrane and in the soluble fraction. The cholesterol and cholesterol esters make up 18.8 percent of the total lipid (Tourtellotte et al., 1963). Cholesterol is an absolute growth requirement for this organism. The work of Smith and Lynn (1958) indicates the 3-beta-hydroxy group, the cyclopentanophenanthrene ring and the 8-carbon side chain of cholesterol are absolute requirements for growth. Even minor modifications in the cholesterol molecule result in a reduction of growth promoting activity or result in inhibition of growth (Smith and Lynn, 1958).

Cholesterol may constitute a very important if not major structural entity of the cell membrane of PPLO (Smith and Rothblat, 1960). Smith (1959) has suggested that cholesterol may play an active role in the cell metabolism of fatty acids. He indicated that cholesterol is utilized by cholesterol esterase with the cofactor participation of lecthin for the synthesis of esters from activated fatty acids. The esters are then transported across the cell membrane to liberate free

fatty acid.

Padgett (1961) suggested that DOC may act as a competitive inhibitor of cholesterol in the cell metabolism. This hypothesis, prompted by the structural similarity between DOC and cholesterol, was substantiated by the fact that saprophytic PPLO (those not requiring cholesterol) were not inhibited by DOC (Padgett and Schoenhard, unpublished data). Evidence contrary to this hypothesis is found in the work of Lester and Hechter (1958) who have shown that organisms not dependent on cholesterol (gram-positive bacteria and some yeasts and molds) are inhibited by DOC, and by Smith and Rothblat (1960) who suggest that since PPLO could not be saturated with cholesterol, other sterols do not compete with cholesterol in the absorbtion process.

Smith (1959) observed that sterols which inhibit growth of PPLO are inhibitory to the cholesterol esterase activity. The mode of inhibition was not explained.

DOC did not inhibit the in vitro growth of the strain of Mycoplasma gallisepticum used in this study. It is interesting that after 5 subcultures in Grumble's medium, the organism acquired a susceptibility to the inhibitory action of DOC and growth could be inhibited by 0.125 mg of DOC per ml of medium (Lund and Schoenhard, unpublished data). The latter response of the culture to DOC is similar to the results obtained by Padgett (1961). Growth conditions and culture history probably play an important role in the susceptibility of PPLO to DOC.

The inability of DOC to act as a therapeutic agent toward the air sac lesions of experimentally produced CRD in chickens appears to be

significant. Padgett and Schoenhard (1959) were able to markedly reduce the recovery time of chickens with CRD, by treating the birds with DOC. As stated above the strain of M. gallisepticum they were working with was susceptible to DOC in vitro. Thus there appears to be a difference in response to DOC between strains of M. gallisepticum.

Many of the investigators working with CRD have suggested that the pathology seen is produced by secondary invaders, particularly Escherichia coli and Pseudomonas aeruginosa. Lester and Hechter (1958) have shown that gram-negative bacteria are not affected by DOC. Since the efficacy of DOC in treating CRD appears to be dependent upon the susceptibility of the M. gallisepticum organism, and it is not effective against the secondary invaders, it would appear that the M. gallisepticum organism is responsible for the pathology seen with CRD. This hypothesis is supported by the work of Smibert et al. (1959), in which they were able to produce all the symptoms of CRD by the inoculation of PPLO into germ-free chickens and turkeys and by the excellent results obtained with the use of tylosin in this study. Tylosin is an antibiotic with an essentially gram-positive bacterial spectrum. It is also highly active against PPLO of swine and avian origin (McGuire et al., 1961).

As shown in Tables 4 and 5, treatment with tylosin resulted in a marked reduction in the number of isolable organisms, the feed per pound of gain decreased (when compared to non-treated controls) and the gross pathology was considerably reduced. Treatment with DOC resulted in no significant reduction in the number of isolable organisms, the feed conversion ratio increased and the gross pathology was not reduced. When the combination of DOC and tylosin was used, the feed conversion ratio was higher (less efficient) than when tylosin was used alone. It

would appear that DOC may place some stress on the birds, since the feed conversion ratio is higher than the non-treated control birds. But this is not consistent with data obtained from other studies (Lund and Schoenhard, unpublished data). In three previous experiments, birds treated with DOC had a higher feed efficiency than the untreated control birds even though the air sac lesions and symptoms were apparently not affected. Padgett (1961) found DOC to be non-toxic to chickens in the dosage used (20 mg per bird).

The immunization procedure of simultaneous injection of whole bacteria with NaOH treated lipopolysaccharide adsorbed on erythrocytes was chosen because of the results obtained by Seigneurin et al. (1962). Seigneurin et al. (1962) were able to protect rabbits against an intraperitoneal injection of 6.5×10^9 E. coli organisms. The use of whole bacteria or the lipopolysaccharide alone did not protect the rabbits.

The protein content of the lipopolysaccharide used in Experiments III and IV was 384 ug per ml or 7.7 percent of the lipopolysaccharide. This value is within the range reported by Nowotny et al. (1953), Fukushi et al. (1964), and Ribi et al. (1961a and b) values from 1.8 percent to 9.7 percent protein.

The total carbohydrate content of the lipopolysaccharide (38 percent) was above the reported values which range from 19.7 percent to 31.0 percent (Nowotny et al., 1963; Fukushi et al., 1964).

If the RNA determination were correct (no interference by other compounds) the 1:10 dilution of the lipopolysaccharide should have contained 80 ug of RNA per ml. Because the RNA standard (80 ug/ml) showed an absorption spectrum typical of RNA with a peak absorption at 260 mu and neither the 1:10 nor the 1:20 dilution of the lipopolysaccharide

showed any increase in absorption (Figure 1) in the range from 240 to 310 mu it was concluded that the lipopolysaccharide did not contain any RNA, or if present it was in very minute amounts.

The LD_{50} of the lipopolysaccharide was 172.8 ug per mouse or 10.7 ug per gm. Nowotny et al. (1963), and Neter et al. (1956), reported LD_{50}'s for the E. coli endotoxin ranging from 150 ug to 420 ug per mouse.

The attempt to immunize chickens against E. coli was undertaken to attain a better understanding of the role of secondary invaders in the production of air sac pathology in CRD. If the immunization of the chickens against E. coli had proven feasible, the next step would have been to prepare a polyvalent vaccine containing the bacteria found most often as secondary invaders. If the invasion of the air sacs by the bacteria other than M. gallisepticum could have been prevented, and typical air sac lesions developed subsequent to inoculation with M. gallisepticum then it would have indicated that M. gallisepticum was primarily responsible for the air sac pathology. The use of such a system for screening drugs for activity against CRD would have been advantageous, since the problem of the secondary invasion could have been circumvented.

The results of the immunization of the chickens were at first very encouraging because all of the immunized birds survived the challenge with the live E. coli organisms.

It was disappointing on necropsy examination to find that the immunization procedure had not prevented the initiation of air sac, pericardium and liver capsule lesions.

It was then decided to use high titer rabbit anti-E. coli serum

in an attempt to passively immunize the chickens against E. coli. The gamma fraction was used initially in order to keep the amount of rabbit protein injected into the chickens at a minimum. When it became apparent that passive immunization of the chickens resulted only in preventing death, and no significant protection against the initiation of air sac, pericardium and liver capsule lesions, the use of whole serum was tried. It was thought that either the protective antibody was in some way denatured during the serum fractionation, or the protective antibody was present in a serum fraction other than the gamma fraction. Because the use of a lethal dose of the E. coli organisms was somewhat unrealistic, that is, the natural exposure to the E. coli organisms is undoubtedly of a lesser amount, the birds were challenged with a minimal infective dose. There was no significant protection afforded by the use of either the gamma fraction or the whole serum.

Protection of the air sacs against bacterial invaders by immunization appears to be a difficult task. McMartin and Adler (1961) were able to immunize chickens against M. gallisepticum by the intranasal inoculation of 300 organisms. However, some local air sac lesions were produced. They were not able to impart passive immunity using an immune serum of 640 agglutination titer. Adler et al. (1960) were able to immunize chickens against M. gallisepticum by the intramuscular injection of an attenuated culture; however, the immunity was short lived, lasting only 2 months. Gross (1956) was able to achieve a protection of 84 percent of the chickens vaccinated by using an E. coli aerosol for vaccination. This method would not be commercially feasible since if M. gallisepticum is present, high losses result from the aerosol exposure to E. coli (Gross, 1956).

SUMMARY

Mycoplasma gallisepticum, an avian PPLO, was not inhibited *in vitro* by deoxycorticosterone. Growth was inhibited by tylosin at the level of 0.01 ug per ml of medium.

Deoxycorticosterone had no observable effect as a therapeutic agent toward the air sac lesions and symptoms of experimentally produced CRD in chickens. Tylosin inhibited the PPLO and the symptoms and pathology of CRD in the chickens were reduced, with a resultant increase in feed conversion efficiency when compared to infected, non-treated control birds. The use of a combination of the two drugs resulted in no greater therapeutic response than that seen with tylosin alone.

The active and passive immunization of chickens against the *E. coli* organism resulted, on challenge with live organisms, in protection against death but in no protection against the fibrinous inflammatory lesions involving the air sacs, liver capsule, and pericardium.

BIBLIOGRAPHY

Adler, H. E., D. McMartin, and M. Shifrine. 1960. Immunization against Mycoplasma infections of poultry. Am. J. Vet. Res. 21:482-485.

Barnes, L. E., E. E. Ose, and F. O. Gossett. 1960. Treatment of experimental PPLO infections in young chickens with tylosin, a new antibiotic. Poult. Sci. 39:1367-1381.

Beckman, J. R., W. R. Dunlop, and W. Staples. 1959. Observations in the prevention of the CRD complex. Poult. Sci. 38:807-816.

Blake, J. T. 1961. Influence of certain improved antibiotic formulations upon the course of CRD in a breeder flock. Poult. Sci. 40:911-918.

Brandly, C. A. 1955. Diagnosis and control of respiratory diseases of poultry. North Am. Vet. 36:644-647.

Boone, M. A., D. J. Richey, and C. L. Morgan. 1957. Effects of antibiotics on egg production when chickens are naturally infected with chronic respiratory disease. Poult. Sci. 36:1340-1344.

Chargaff, E., and J. N. Davidson. 1955. The nucleic acids, vol. 1. p. 267-302. Academic Press, Inc., New York.

Chattaway, F. W., J. D. Townsley, and A. J. L. Barlow. 1963. On the mode of action of 11-deoxycorticosterone on the metabolism of Trichophyton rubrum. J. Gen. Bacteriol. 30:261-262.

Cover, M. S. 1955. The therapeutic use of antibiotics for chronic respiratory disease in three laying flocks. Poult. Sci. 34:686-690.

Cowgill, R. W. and A. B. Pardee. 1957. Experiments in biochemical research techniques, p. 176. John Wiley and Sons, Inc., New York.

Delaplane, J. R., and H. O. Stuart. 1943. The propagation of a virus in embryonated chicken eggs causing a chronic respiratory disease of chickens. Am. J. Vet. Res. 4:325-332.

Edward, D. G., and A. D. Kanarek. 1960. Organisms of the pleuropneumonia group of avian orgin: their classification into species. Ann. N.Y. Acad. Sci. 79:696-702.

Fahey, J. E., and J. F. Crawley. 1954. Studies on chronic respiratory disease of chickens, III. Egg transmission of a pleuropneumonia-like organism. Can. J. Comp. Med. 19:281-286.

Fahey, J. E., and J. F. Crawley. 1955. Studies on chronic respiratory disease of chickens, VI. The effects of antibiotics on the clinical and serological course of CRD. Can. J. Comp. Med. 19:281-286.

Fukushi, K., R. L. Anacher, W. T. Haskins, M. Landy, H. C. Milner, and E. Ribi. 1964. Extraction and purification of endotoxin from Enterobacteriaceae: a comparison of selected methods and sources. J. Bacteriol. 87:391-400.

Gross, W. B. 1956. Chronic respiratory disease of chickens and infectious sinusitis of turkeys: Escherichia coli as a complicating factor. Ph.D. Thesis, University of Minnesota, Minneapolis.

Gross, W. B. 1961. The effect of chlortetracycline, erythromycin, and nitrofurans as treatments for experimental "air sac disease". Poult. Sci. 41:833-841.

Heishman, J. O., N. O. Olson, and C. J. Cunningham. 1962. Control of CRD IV. The effect of low calcium diet and high concentration of chlortetracycline on the isolation of Mycoplasma from experimentally infected chicks. Avian Dis. 6:165-170.

Kabat, E. A., and M. M. Mayer. 1961. Experimental immunochemistry. P. 833-835. Charles C. Thomas, Pub., Springfield.

Kiser, J. S., F. Popken, and J. Clemente. 1960. Antibiotic control of an experimental Mycoplasma gallisepticum (PPLO) infection in chickens. Ann. N.Y. Acad. Sci. 79:593-607.

Lecce, J. G., and F. G. Sperling. 1955. Chronic respiratory disease. III. The effect of treatment of the pleuropneumonia-like organism flora of avian tracheas. J. Am. Vet. Med. Assoc. 127:54-56.

Lester, G., and O. Hechter. 1958. Effect of deoxycorticosterone on the growth of microorganisms. J. Bacteriol. 76:365-367.

Lynn, R. J., and P. F. Smith. 1960. Chemical composition of PPLO. Ann. N.Y. Acad. Sci. 79:493-498.

Markam, F. S., and S. C. Wong. 1952. Pleuropneumonia-like organisms in the etiology of turkey sinusitis and chronic respiratory disease of chickens. Poult. Sci. 31:902-904.

Maxwell, E. S., J. S. McGuire, and G. M. Tomkins. 1960. Antibiotic effect of steroids on Saccharomyces fragilis and the isolation of a resistant mutant. J. Bacteriol. 80:1-6.

McGuire, J. M., W. S. Boniece, C. E. Higgens, M. M. Hoehn, W. M. Stark, J. Westhead, and R. N. Wolfe. 1961. Tylosin, a new antibiotic. 1. Microbiological studies. Antibiot. Therap. 11:320.

McMartin, D. A., and H. E. Adler. 1961. An immunological phenomenon in chickens following injection with Mycoplasma gallisepticum. J. Comp. Path. 71:311-323.

Morris, D. L. 1948. Colorimetric determination of total carbohydrates. P. 33-34. In A. C. Neish (ed.), Analytical methods for bacterial fementations. Natl. Res. Coun. Can., Saskatoon.

Nelson, J. B. 1935. Cocco-bacilliform bodies associated with an infectious fowl coryza. Science. 82:43-44.

Neter, E., O. Westphal, O. Luderitz, E. A. Gorzynski, and E. Eichenberger. 1956. Studies on enterobacterial lipopolysaccharides, effects of heat and chemicals on erythrocyte-modifying, antigenic, toxic, and pyrogenic properties. J. Immunol. 76:377-385.

Nowotny, A. M., S. Thomas, O. S. Duron, and A. Nowotny. 1963. Relation of structure to function in bacterial O antigens I. Isolation methods. J. Bacteriol. 85:418-424.

Olesiuk, O. M., and H. VanRoekel. 1959. The effects of antibiotics on experimental chronic respiratory disease in chickens. Avian Dis. 3:457-470.

Olesiuk, O. M., and H. VanRoekel. 1960a. Transmission of chronic respiratory disease in chickens. Avian Dis. 4:348-368.

Olesiuk, O. M., and H. VanRoekel. 1960b. Pathological and immunological observations concerning avian pleuropneumonialike organisms. Ann. N.Y. Acad. Sci. 79:727-740.

Olesiuk, O. M., and H. VanRoekel, and L. P. Beninato. 1957. Influence of chemotherapeutic agents on experimental chronic respiratory disease in chickens and turkeys. Poult. Sci. 37:383-395.

Olson, N. O., D. C. Shelton, and J. O. Heishman. 1959. Control of chronic respiratory disease I. The effects of chlortetracycline (CTC) and terephthalic acid (TPA). Avian Dis. 3:443-451.

Padgett, G. A. 1961. The in vitro and in vivo response of Mycoplasma gallisepticum to deoxycorticosterone. M.S. Thesis, Michigan State University, East Lansing.

Padgett, G. A., and D. E. Schoenhard. 1959. Effect of deoxycorticosterone on the agent of chronic respiratory disease. M.S.U. Vet. 20:27-31.

Perek, M., and K. Perek. 1958. Studies of the effect of dihydrostreptomycin propylene-glycol spray on chronic respiratory disease in chickens. Avian Dis. 2:269-278.

Price, K. E., Z. Zolli, Jr., W. B. Hardie, and M. J. Gallian. 1957. Respiratory tract flora in CRD and effect of antibiotics in the feed. Poult. Sci. 36:219-225.

Price, K. E., Z. Zolli, Jr., W. B. Hardie, and R. Owens. 1958. The influence of penicillin and oxytetracycline in the ration on mortality, lesions and predominating microflora in experimental CRD. Poult. Sci. 37:472-478.

Reed, L. J., and H. Muench. 1938. A simple method of estimating fifty percent endpoints. P. 66-70. In C. H. Cunningham. A laboratory guide in virology. Burgess Publishing Company, Minneapolis.

Ribi, E., W. T. Haskins, M Landy, and K. L. Milner. 1961a. Preparation and host-reactive properties of endotoxin with low content of nitrogen and lipid. J. Exptl. Med. 114:647-663.

Ribi, E., W. T. Haskins, M. Landy, and K. L. Milner. 1961b. Symposium on bacterial endotoxins I. Relationship of chemical composition to biological activity. Bacteriol. Rev. 25:427-436.

Roberts, R. S. 1949. The endotoxin of Bacti. coli. J. Comp. Path. Therap. 59:284-303.

Rosenberg, D. W., W. D. Woodward, and A. E. Kline. 1962. Effect of two nitrofurans and zoalene on poultry condemnations due to air sac infections. J. Am. Vet. Med. Assoc. 141:958-959.

Seigneurin, R., R. Magnin, and Mme J. Fils. 1962. Essais d'immunisation du lapin contre les E. coli 111B4 enteropathogenes par injection simultanee du lipopolysaccharide specifique et du germe correspondant chauffe 1 heure a 56°. Ann. Inst. Pasteur (Par.). 102:44-48.

Smibert, R. M., M. Forbes, J. E. Faber, A. B. Gabuten, and H. M. DeVolt. 1959. Studies on "air sac" infection in poultry. 1. Infection of germ free turkeys with Mycoplasma gallinarum (avian PPLO) from sinal exudate and broth cultures. Poult. Sci. 38:676-684.

Smith, P. F. 1959. Cholesterol esterase activity of pleuropneumonialike organisms. J. Bacteriol. 77:682-689.

Smith, P. F. 1960. Nutritional requirements of PPLO and their relation to metabolic function. Ann. N.Y. Acad. Sci. 79:508-520.

Smith, P. F., and R. J. Lynn. 1958. Lipid requirements for the growth of pleuropneumonialike organisms. J. Bacteriol. 76:264-269.

Smith, P. F., and G. H. Rothblat. 1960. Incorporation of cholesterol by pleuropneumonia-like organisms. J. Bacteriol. 80:842-850.

Taylor, J. R. E., and J. Fabricant. 1957. Studies on the isolation of the pleuropneumonialike organism of chronic respiratory disease of fowls. Cornell Vet. 47:112-125.

Tourtellotte, M. E., R. G. Jensen, G. W. Gander, and H. J. Morowitz. 1963. Lipid composition and synthesis in the pleuropneumonia-like organism Mycoplasma gallisepticum. J. Bacteriol. 86:370-379.

VanRoekel, H., O. M. Olesiuk, and H. A. Peck. 1952. Chronic respiratory disease of chickens. Am. J. Vet. Res. 13:252-259.

Wasserman, B., V. J. Yates, and D. E. Fry. 1954. On so-called air-sac infection. Poult. Sci. 33:622-623.

White-Stevens, R., and H. G. Zeibel. 1954. The effect of chlortetracycline (Aureomycin) on the growth efficiency of broilers in the presence of chronic respiratory disease. Poult. Sci. 33:1164-1174.

Whitehill, A. R., and J. S. Kiser. 1961. The effectiveness of several tetracyclines in the control of an experimental PPLO (Mycoplasma gallisepticum) infection in chickens. Avian Dis. 5:188-195.