BIOLOGY OF *ERYSIPELOTHRIX RHUSIOPATHIAE*

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1969

Submitted to the Faculty of the Graduate College
of the Oklahoma State University
in partial fulfillment of the requirements
for the Degree of
MASTER OF SCIENCE
May, 1975

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BIOLOGY OF ERYSIPELOTHRIX RHUSIOPATHIAE

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ACKNOWLEDGEMENTS

The author is sincerely grateful to Dr. Richard E. Corstvet for his patience, guidance and encouragement throughout the course of this study and the preparation of this thesis.

The author expresses his sincere appreciation to other members of his advisory committee, Dr. Elizabeth T. Gaudy and Dr. George V. Odell for their helpful discussion, guidance, suggestions, and critical review of this thesis.

Special thanks are extended to Dr. Jak K. Chen for many suggestions in the chromatography experiments and to Mr. Jim Powell for his assistance in the preparation of this thesis.

Special recognition is extended to my parents and to my wife, Bao-shin, for their understanding, encouragement, and confidence which have made the achievement of this study possible.

TABLE OF CONTENTS

Chapter | Page

I. INTRODUCTION 1

II. ASSAY OF TOXINS PRODUCED BY E. RHUSIOPATHIAE 7

Materials and Methods 7
Organism 7
Media 7
Chemicals 10
Preparation of Washed Cells 10
Titration of Bacteria 10
Concentrating the Samples for Assay of Toxin 11
Assay to Toxin 12
Chromatography Studies 12
Results 14
Selection of Media 14
Assay of Toxin(s) Produced in Liquid Culture 16
Discussion 26

III. STUDIES ON THE RELATIONSHIP BETWEEN THE VIRULENCE OF E. RHUSIOPATHIAE AND THEIR PROPHAGE 32

Materials and Methods 32
Bacteria 32
Media 32
Chemicals 33
Selection of the Most Ultraviolet Light-sensitive Strain 33
Demonstration of Bacteriophage by Induction of Cell Lysis 34
Isolation of Phage-Susceptible Organisms by a Curing Method 35
Isolation of Bacteriophage 36
Preparation of Filtrate for Phage Stock 36
Preparation of Seed Cells 37
Bacteria Titer 37
Results and Discussion 37
Selection of the Most UV Sensitive Strain . . . 37
Demonstration of Bacteriophage by Induction of Cell Lysis 39
Isolation of Bacteriophage from Virulent E. rhusiopathiae Strains 40

Chapter Page

IV. ISOLATION OF L-PHASE E. RHUSIOPATHIAE FROM INFECTED TURKEYS 43

Bacteria 43
Experimental Turkeys 43
Bacteriological Technique 44
Plate Agglutination Test 45
Antiserum 45
Media 46
Isolation of L-form of E. rhusiopathiae 46
Results and Discussion 47

A SELECTED BIBLIOGRAPHY 51

LIST OF TABLES

Table Page

I. Results of the Inoculation of Various Media with E. rhusiopathiae Strain 273 15

II. Results of the Inoculation of Media Containing Casitone as the Basic Nutrient (Medium III) with E. rhusiopathiae Strain 273 17

III. Results of the Inoculation of Media Containing Proteose Peptone No. 3 as Basic Nutrient (Medium II) with E. rhusiopathiae Strain 273 18

IV. Assay for Toxin Production by E. rhusiopathiae in Various Liquid Media 19

V. Result of Selection of the Most UV Sensitive E. rhusiopathiae Strain 38

VI. Results of the Isolation of L-Phrase and Parent Form from E. rhusiopathiae From Infected Turkey's Blood 48

LIST OF FIGURES

Figure		Page
1.	Analysis by Gel Filtration of BHl Broth *E. rhusiopathiae* Culture for the Presence of a Toxin on Sephadex G-100 Column at Room Temperature	21
2.	Gel Filtration of Noninoculated BHl Broth on Sephadex G-100 Column	23
3.	Analysis by Gel Filtration of BHl Broth E. rhusiopathiae Culture for the Presence of a Toxin on Sephadex G-100 Column	24
4.	Gel Filtration of Noninoculated BHl Broth on Sephadex G-100 Column	25

CHAPTER I

INTRODUCTION

Erysipelothrix rhusiopathiae was first isolated by Robert Koch from mice which developed septicemia after being fed putrid meat. In 1885 and 1886 Loeffler demonstrated that swine erysipelas was caused by a bacillus identical to, or closely resembling, that isolated from the septicemic mice of Koch. In 1885, Smith first reported the isolation from a sick pig of micro-organisms closely resembling the bacillus of swine erysipelas. Jarosch (1905) was the first to recognize this disease, isolating the organism from a turkey. Beaudette and Hudson (1936) were first to call attention to the economic significance of the disease in turkeys on the American continent. Madsen (1937), Van Roekel, Bullis, and Clarke (1938) and Rosenwald and Dickinson (1939) also reported natural outbreaks of *E. rhusiopathial* infection in turkeys later. According to various published reports, *E. rhusiopathiae* has been isolated not only from chickens, goslings, pheasants, parakeets, peacocks, quail, white stork and herring gull, and a number of wild birds, but also from swine, sheep, cattle, marine and fresh water fish, mink, horse, dog, mice, rat, chipmunk, porpoise, and man (Waller, 1939; Woodbine, 1950; Kilian et al., 1958; Blackmore and Gallagher, 1964; Baillie et al., 1970; Levine, 1965; Geraci et al., 1966; Poels, 1919; Urbain, 1963; Greener, 1939; Jarmai, 1920; Bourgeois, 1944; Van Bemmel et al., 1960; Van Es and McGrath, 1936; Stiles, 1944; Drake and Hall,

1947; Grey, 1947; Connell, 1954; Seibold and Neal, 1956). Within the last three decades *E. rhusiopathiae* has become recognized as important to animal health in America.

Pathogenesis of *E. rhusiopathiae* in swine includes three recognizable forms: the acute or septicemic type, the chronic form with endocarditis and arthritis, and the skin form, or "diamond skin disease," which may be followed by gangrenous dermatitis. In turkeys, infected birds harbor the organism in various tissues. Characteristic of the acute infection in turkeys is a high temperature, septicemia and sudden death with a high mortality varying from 25 to 50 per cent (Corstvet et al., 1968; Corstvet, 1967; Rosenwald and Corstvet, 1972). However, the relationship between the host and the parasite is still unclear. Van Es and McGrath (1936) cited Nocard and Leclainche as saying in 1903 that "at the present state of our knowledge it is impossible to explain the mysterious behavior of the contagion." This is still true at the present time, especially in its mechanism of causing the syndrome in infected animals.

White and Puls (1969) reported that a high pressure jet infection of cell-free crude extract of *E. rhusiopathiae* into the knee-joint of rabbits stimulated an acute, mild inflammatory reaction. Additional injections at three-day intervals induced a chronic arthritis. White, Puls, and Mirikitani (1971) further studied the characteristics of the cell-free extract and found that the extract after intravenous inoculation can also induce rabbit arthritis. The toxic material was isolated from the cell wall in their experiment. White and Verwey (1970) isolated an immunogenic particle from culture supernatant fluids of *E. rhusiopathiae* and found that the protective antibody was the

lipid and protein part of the particle. Further studies of the particle were done by the same authors (1970) revealing that the particle was not a fragment of the aggregated cell wall or cell membrane of the organism but rather a glycolipoprotein. In *Bacillus anthracis*, a specific immunogen that induces a protective antibody was isolated; this was related immunologically to the lethal toxin and was presumably the same as the toxic material produced by this organism (Smith et al., 1955). Finally, Mahlandt et al. (1966) demonstrated that the protective antigen is one component of the anthrax toxin. It is suspected that the same phenomenon will also be found in *E. rhusiopathiae* infection. Corstvet (1970) reported that when the population of *E. rhusiopathiae* exceeded 10^4 organisms per ml of blood, the bird succumbed to the infection. Infected turkeys die with a high concentration of bacteria in the blood. This phenomenon is the same as in *B. anthracis* infection in which a lethal exotoxin is produced. On the other hand, death of *E. rhusiopathiae* infection in turkeys usually occurs acutely. This may also indicate that the organism produces a toxin responsible for the death of infected animals. The fact that a high concentration of bacteria is required for the death of the animals must be due to a low concentration of toxin produced, or to the low toxicity of the toxin. This study was mainly concerned with the existence of the toxic materials. *In vitro* attempts were made to demonstrate the toxic materials.

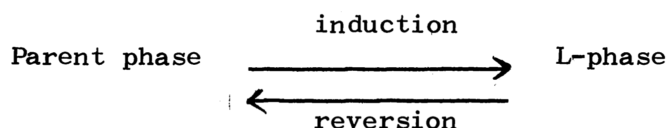
The importance of Freeman's remarkable discovery (Freeman, 1951, Freeman and Morse, 1952) that certain strains of *Corynebacterium diphtheriae* rendered lysogenic by a particular bacteriophage are capable of producing diphtheria toxin cannot be overstated. This discovery

paved the way for much of what is now known concerning phage-host relationship in production of certain bacterial toxins. Later Kjems (1955, 1960) and Krause (1957) demonstrated that 20-50 per cent of randomly selected Lancefield group A Streptococcus sp. strains were lysogenic. Matsuda and Barksdale (1966) demonstrated that a phage and erythrogenic toxin production were interrelated in a manner similar to that observed for C. diphtheriae. The relationship between toxigenicity of staphylococcus and its prophage was also reported by Blair and Carr (1961). They noted that following lysogenization with a bacteriophage obtained from a known toxin producing strain, two of three strains tested became toxigenic. The evidence now suggests that at least three toxins are in some manner phage-mediated (Casman, 1965). Groman (1966) reported that the conversion of toxigenicity of C. diptheriae is bacteriophage-specific.

Bacteriophage involved in change from nontoxigenicity to toxigenicity of Clostridium botulinum type C has been reported by Eklund et al. (1970). Eklund et al. (1972) also reported that production of C. botulinum type D toxin is mediated by a certain type of bacteriophage. C. novyi is divided into four types designated as types A through D based on the production of toxins and other biologically active substances. C. botulinum includes a very heterogeneous group of strains that are divided into types A through G based on the antigenic specificity of neurotoxins that are produced (Solomon et al., 1971); Holderman and Brooks, 1970). The strains of C. botulinum types C and D share some of the same characteristics possessed by C. novyi type A (Holderman and Brooks, 1970, Smith and Holderman, 1968). The difference between the species is the toxins produced.

Eklund et al. (1974) reported that interspecies conversion of *C. botulinum* type C to *C. novyi* A can be mediated by a certain bacteriophage. It is unknown whether the *E. rhusiopathiae* phage is involved in the toxigenicity mediation of this bacteria. An attempt was also made to investigate this relationship.

Another objective of this research was to determine the phase of *E. rhusiopathiae* in the carrier state of infected turkeys. Normally, a microbial population will be in the parent phase (with bacterial wall). Under certain conditions, a proportion of the bacteria in the L-phase may form a complete and rigid cell wall again. Induction and reversion might be depicted as being a kind of dynamic equilibrium:



Sadler and Corstvet (1965) found that *E. rhusiopathiae* infected turkeys remained in a carrier state and showed no clinical signs or gross pathologic lesions. *E. rhusiopathiae* could be isolated from liver, bone marrow, and digestive tract during the carrier state. Procaine penicillin and erythromycin could not eliminate the carrier state (Corstvet and Sadler, 1965; Corstvet et al., 1970). However, the factors that enter into production of such a carrier state have not been ascertained.

It is suspected that interconversion between parent phase and L-phase contributed to this long persistance in infected turkeys. In addition, morphologic entities resembling the L-phase of *E. rhusiopathiae* have been observed *in vivo* by the fluorescent antibody technique (Corstvet, unpublished data). Further study to prove the existence of the L-phase in infected turkeys' blood and the

cultivation of the L-phase *in vitro* would be worthwhile in order to study factors that produce the carrier state in infected birds. Such a study would further explore the role of the L-phase bacteria in the host-parasite relationship.

CHAPTER II

ASSAY OF TOXINS PRODUCED BY *E. RHUSIOPATHIAE*

Materials and Methods

Organism

Two virulent strains of *E. rhusiopathiae* were used. They are strains 273 and 5483 from our laboratory stock. Stock cultures were grown in embryonal chicken yolk sac and stored in the freezer at -70°C. The original culture of strain 273 was from the University of California, Davis, while strain 5483 was from 273 and passed through turkeys three times. The virulence of both strains was tested by infection of three four-week old turkeys with 10^{6} cells which had been grown in the yolk sac of embryonal chicken eggs. The yolk was diluted with trypticase soy broth for the inoculation of the turkeys. All three turkeys were dead within 72 hours with gross lesions typical of *E. rhusiopathiae* infection indicating that the organisms were virulent. Only strain 273 was used in the studies of medium selection.

Media

The following media were used in attempts to cultivate *E. rhusiopathiae*:

(1) Medium I: The first medium was brain heart infusion broth (Difco). The brain heart infusion broth was prepared as suggested by

the manufacturer. In addition, five per cent (v/v) sterile horse serum and one per cent (w/v) yeast autolysate (Pfizer) was added to the broth before use.

(2) Medium II: The second medium was proteose peptone No. 3 broth to which was added 0.01 per cent (w/v) glucose, and 0.25 per cent (w/v) sodium chloride. This was prepared by dissolving 1 g proteose peptone No. 3 and 0.25 g sodium chloride in 90 ml deionized water. This was then autoclaved at 121°C for 15 minutes. After cooling, 10 ml of 0.1 per cent (w/v) glucose sterilized by filtration was added.

(3) Medium III: Casitone (Difco) broth to which was added 0.01 per cent (w/v) glucose and 0.25 per cent (w/v) sodium chloride. Preparation of the medium was the same as Medium II.

(4) Medium IV: The casamino acids medium, which has been used previously for the production of anthrax exotoxin, was prepared as described by Belton and Strang (1954).

(5) Medium V: The fifth medium was a modification of the minimal medium which was prepared by David and Mingioli (1950) for Escherichia coli and contained 10 g casamino acids and 5 g glucose per liter. For the preparation of the minimal medium, Davis and Mingioli's suggestion was followed. In addition to the minimal medium, the preparation procedures are described as follows:

To each 95 ml of the minimal medium, 1 g of casamino acids was added. The solution was then autoclaved at 121°C for 15 minutes. After cooling, 5 ml of 10 per cent (w/v) glucose sterilized by filtration was added and the medium was stored at 4°C until used.

(6) Medium VI: This was the same as Medium V, with the

exception that trypticase (Difco) was used instead of the casamino acids.

(7) Medium VII: The seventh medium was tissue culture medium 199, a product of Difco Laboratories, and was prepared as suggested by the manufacturer.

(8) Medium VIII: The eighth medium was the same as Medium VII, with the exception that 5 per cent (v/v) rabbit red blood cell lysate was added. Preparation of the red blood cell lysate was as follows: Red blood cells from a rabbit were spun down by centrifugation and lysed by suspension of cells in 20 volumes of 0.02 M phosphate saline buffer pH 7.2. The cell membranes were separated by centrifugation at 20,000 x g for 20 minutes. The supernatant was sterilized by filtration through a 0.2 μ membrane filter (millipore) and kept at $4^{o}C$ until used.

(9) Medium IX: The ninth medium was also the same as Medium VIII, with the exception of the addition of serum dialysate. A 15 cm length of dialysis sac (pore size 24 A^{o} diameter) with a knot in one end was sterilized by submerging it in water and autoclaving at $121^{o}C$ for 15 minutes. Then the sac was removed with sterile forceps and 20 ml of horse serum was injected into the open end. A knot was then tied in the open end and the sac was suspended in a 300 ml flask containing 200 ml of tissue culture medium 199 (Difco). The medium was stirred at a moderate speed with a magnetic stirrer for 12 hours at $4^{o}C$. The dialysis sac and its contents were discarded. The medium in the flask was saved for use as Medium IX.

Blood agar plates were prepared by adding 5 per cent (v/v) bovine blood into heart infusion agar (Difco) and enriching with 5 per cent

(v/v) horse serum. The preparation of the heart infusion agar followed the manufacturer's suggestion. Five per cent bovine blood and 5 per cent horse serum were added before pouring the plates.

Chemicals

The following chemicals were purchased from Mallinckrodt Chemical Company: potassium hydroxide (KOH), potassium phosphate monobasic (KH_2PO_4), ammonium sulphate $(NH_4)_2SO_4$, ferrous sulphate ($FeSO_4 \cdot 7H_2O$), sodium bicarbonate ($NaHCO_3$), cobalt sulphate ($CoSO_4 \cdot 7H_2O$), calcium chloride ($CaCl_2 \cdot 1H_2O$) and magnesium sulphate ($MgSO_4 \cdot 7H_2O$) were obtained from J. T. Baker. Acridine orange, sodium citrate ($Na_3C_6H_5O_72H_2O$) and manganese sulphate ($MnSO_4H_2O$) were obtained from Fisher Scientific Company. Uracil was obtained from Matheson, Colman and Bell. Adenine, d-glutamine, guanine, and thiamine HCl were obtained from Sigma Chemical Company. Glycine and Aquacide were Calbiochem products.

Preparation of Washed Cells

Cells were grown in 10 ml of brain heart infusion broth (Difco) containing 10 per cent horse serum and 1 per cent yeast autolysate (Pfizer) at $37^{\circ}C$ for 12 hours and harvested by centrifugation. These cells were washed three times by suspending the cells in 0.01 M phosphate buffered saline pH 7.2 and collected by centrifugation.

Titration of Bacteria

Six 0.01 ml aliquots of a 10-fold dilution of the bacterial cells suspension to be titrated were put on 5 per cent bovine blood agar

plate enriched with 5 per cent horse serum. The plates were kept in a candle jar and incubated at 37°C. Aliquots of less than 30 colonies were counted 48 hours after incubation and the bacterial titer was calculated. Phosphate buffered saline (pH 7.2,0 .1M) was used as diluent.

Concentrating the Samples for Assay of Toxin

Two methods were employed for concentrating samples:

(1) Using Aquacide

Samples were concentrated by means of absorption by aquacide of low molecular weight substances which could diffuse from the dialysis sac.

The dialysis sac with pore size of 24 A° diameter was tied in a tight knot at one end and put in a 500 ml flask. The flask was filled with 300 ml water and autoclaved at 121° C for 30 minutes. After cooling, the sterile dialysis sac was carefully removed from the flask with a sterile forcep. Twenty ml of the sample to be concentrated was injected into the dialysis sac by means of a syringe equipped with a 20 gauge needle. The dialysis sac was covered with aquacide (non-sterile) after the open end of the tubing had been tied in a tight knot. The covered aquacide was removed when approximately 2 ml of the sample could be removed from the sac. Concentration of the obtained sample was considered 10 fold of the original liquid culture.

(2) Using Lyophilization

Samples were concentrated by sublimation of water from the frozen state under high vacuum. Each sample to be concentrated was poured into a round bottom flask with at least three times the volume of the

sample. The flask was rested in a pan containing dry ice and acetone and then tilted and rotated until the solution was frozen as a smooth coat covering the inside of the flask. The sample was lyophilized while still in the flask for about 12 hours. A 10-fold concentrated sample was obtained by reconstituting the dry sample with a volume one-tenth that of the original sample of 0.01 M phosphate buffered saline (pH 7.2).

Assay to Toxin

The ability to kill mice was used to assay for the presence of toxin in the sample. For the assay, 0.5 ml of each sample to be tested was injected by various routes into each of the mice tested by means of a 0.5 cc disposable syringe equipped with a 25 gauge 5/8 inch needle. Each of the mice injected with each sample was observed for three days. The liver, spleen, and blood of any mouse dying during this three-day period were cultured on blood agar plates to rule out any bacterial infection. All plates were incubated for 48 hours at $37^{o}C$ in a candle jar. Three mice were used for each test and the results were considered negative if all the animals survived three days. For assay of the sample obtained by column chromatography, five mice weighing 25-30 g, were used for each test and the injected mice were also observed for three days.

Chromatography Studies

Each column was packed in a vertical glass tube (2.4 cm internal diameter) across which was fused a sintered-glass disk to support the gel. A layer of glass wool of 0.5 cm depth prevented blockage of the

disk.

Sephadex-gel-filtration media G-100 and G-25 (Pharmacia, Fine Chemicals) were suspended in 0.02M NH_4HCO_3, and allowed to swell for two days and kept at 4^oC until used. Suspensions of the gel were deaerated under reduced pressure before use.

Each column was prepared at room temperature by pouring a thin slurry of the gel particles into 0.02M NH_4HCO_3, and at the same time allowing any excess of liquid to percolate through the growing gel bed. The addition of gel was continued until a bed height of 67 cm for G-100 and 87 cm for G-25 was reached. Then a solvent reservoir was connected to top of the column and the flow of 0.02M NH_4HCO_3 buffer maintained at a rate of approximately 14.1 ml/hr for two days. By this time the bed had settled to a constant height of 64 cm for G-100 and 87 cm for G-25 column.

Chromatography experiments were performed both at room temperature and in a 4^oC cold room. For the experiment performed in the cold room, the same column used at room temperature was moved to the cold room and equilibrated with 0.02 M NH_4HCO_3 for 48 hours.

A sample of 10 ml of the 10-fold concentrated (by lyophilization) sample was applied to the top of the G-100 column. Fractions of 5.2 ml were collected and the absorbance of each was measured at 280 nm in a Coleman 101 Spectrophotometer. Each pooled fraction was freeze-dried soon after the peak was eluted. The freeze-dried samples were kept at 0^oC about one week before use. For further purification of small molecular weight peaks, the pooled fractions were reconstituted and refractionated on a G-25 column.

Results

Selection of Media

Each 10 ml volume of the media (pH 7.3-7.4) to be investigated was delivered into sterile screw cap tubes and inoculated with 0.1 ml of a 12 hour culture of strain 273 that had been washed three times in 0.01 M phosphate buffered saline (pH 7.2). The tubes were incubated statically at 37°C. An initial titer of bacteria in all of the inoculated tubes was obtained by titration of only one of the tubes. Since all tubes were inoculated with 0.1 ml inoculum, the initial titer of organisms in all tubes was reported as the same as the titrated tubes. After 24 to 34 hours incubation, the titer of each tube showing a visible increase in turbidity was determined. Tubes titrated after 24 hours were again titrated after 34 hours incubation. The results are given in Tables I, II and III.

No visual increase in turbidity was found in any of the media used as shown in Table I. In order to allow adaptation of the organism to defined media, 0.5 ml of a 24 hour culture of each of the defined medium tested was transferred to fresh media of the same kind (10 ml in screw cap tubes) and incubated under the same conditions. No visual change in turbidity was found 24 hours after transfer from the first 24-hour culture. The culture titer was not determined since no increase in turbidity was observed.

Since no culture showed any increase in turbidity, it was concluded that neither strain 273 nor strain 5483 grew on media IV, V, VI, VIII or IX, or on Medium VII supplemented with NAD^+ or $(NH_4)_2SO_4$ and $MnSO_4$.

TABLE I

RESULTS OF THE INOCULATION OF VARIOUS MEDIA WITH E. RHUSIOPATHIAE STRAIN 273

Media	Pass I	Pass II
	Change in Turbidity of Medium After 24 Hours Incubation	Change in Turbidity of Medium 24 Hours After Transfer from Pass I
Medium IV	-	-
Medium V	-	-
Medium VI	-	-
Medium VII	-	-
Medium VIII	-	-
Medium IX	-	-
Medium VII NAD	-	-
Medium GII + $(NH_2)SO_4$ + $MnSO^4$	-	-

-: No Change of Turbidity Observed Macroscopically.

Comparison was between Pass I and Pass II of the same medium and not among each of the different media.

Tables II and III reveal that E. rhusiopathiae grew in media containing casitone and proteose peptone No. 3 better than in the defined media. A slight increase in turbidity indicated that growth did occur in both cases. Although culture titers at 0 hours and 34 hours post-inoculation showed no significant increase, Table II suggested that casitone containing media supported with cystine and sodium thioglycolate resulted in better growth. This medium was chosen for toxin production. Proteose peptone No. 3 supported with sodium thioglycolate and cystine was also chosen for testing the production of toxin. A medium containing casitone, supplemented with tryptophen, was also used for toxin production to test the stimulation of toxin productivity by this amino acid.

Assay of Toxin(s) Produced in Liquid Culture

Four media were employed in the attempt to assay exotoxin production. They are:

a. Brain heart infusion broth containing 5 per cent horse serum (v/v) and 1 per cent (w/v) yeast autolysate (Pfizer).

b. Casitone broth containing 0.05 per cent (w/v) cystine and 0.05 per cent (w/v) sodium thioglycolate.

c. Casitone broth containing 0.05 per cent (w/v) sodium thioglycolate and 0.05 per cent (w/v) tryptophan.

d. Proteose peptone No. 3 containing the same amount of cystine, sodium thioglycolate, and yeast autolysate. Media b, c, and d contained 0.1 per cent (w/v) glucose and 0.5 per cent (w/v) sodium chloride. The results are shown in Table IV. No toxicity for mice was found in any of these four media under the different growth conditions and injection routes.

TABLE II

RESULTS OF THE INOCULATION OF MEDIA CONTAINING CASITONE AS THE BASIC NUTRIENT (MEDIUM III) WITH E. RHUSIOPATHIAE STRAIN 273

Media No.	Component of Medium	Change in Turbidity at 24 Hours	0 Hour	34 Hours	64 Hours
I	Medium III + Sodium Thioglycolate	+	8×10^{6}	1.6×10^{7}	1.6×10^{6}
II	Medium III + Sodium Thioglycolate + Cystine	+	8×10^{6}	1.7×10^{8}	1.52×10^{6}
III	Medium III + Sodium Thioglycolate + Yeast Autolysate	+	8×10^{6}	1.1×10^{7}	1.76×10^{6}
IV	Medium III + Cystine + Yeast + Autolysate	+	8×10^{6}	6.9×10^{7}	9.7×10^{5}
V	Medium III + Yeast Autolysate	+	8×10^{6}	5.0×10^{6}	9.5×10^{6}
VI	Medium III only	+	8×10^{6}	1.0×10^{7}	1.9×10^{6}

Medium III: Component and preparation -see Materials and Methods
Sodium Thioglycolate: 0.05% (w/v) Cystine: 0.05% (w/v) Tryptophan: 0.05% (w/v)
Final pH: 7.3 - 7.4
Final pH: 7.0 - 7.2 (at 64 hours)
+ Increase in Turbidity
Each tube contains 10.0 ml of liquid medium. Inoculum was the washed 12-hour culture

TABLE III

RESULTS OF THE INOCULATION OF MEDIA CONTAINING PROTEOSE PEPTONE NO. 3 AS BASIC NUTRIENT (MEDIUM II) WITH E. RHUSIOPATHIAE STRAIN 273

Media No.	Component of Medium	Change of Turbidity at 24 Hours	Culture Titer	
			0 Hour	24 Hour
1	P. P. No. 3 + Sodium Thioglycolate + Cystine + Yeast Autolysate + Tryptophan	+	5.5×10^6	10^6
2	P. P. No. 3 + Sodium Thioglycolate + Cystine + Tryptophan	+	"	10^6
3	P. P. No. 3 + Sodium Thioglycolate + Cystine	+	"	10^6
4	P. P. No. 3 + Sodium Thioglycolate + Tryptophan	+	"	10^6
5	P. P. No. 3 + Sodium Thioglycolate	+	"	10^6
6	P. P. No. 3 + Cystine	+	"	1.9×10^7
7	P. P. No. 3 + Cystine + Tryptophan	+	"	1.8×10^7
8	P. P. No. 3 + Cystine + Tryptophan + Yeast Autolysate	+	"	8×10^6
9	P. P. No. 3 + Sodium Thioglycolate + Yeast Autolysate	+	"	9×10^6
10	P. P. No. 3 + Tryptophan	+	"	1.4×10^7

Medium II: Component and preparation see Materials and Methods
P. P. No. 3: Proteose Peptone No. 3 (Difco)
Sodium thioglycolate: 0.05%(w/v) Cystine; 0.05% (w/v) Tryptophan: 0.05% (w/v)
Initial pH: 7.3-7.4 Final pH: 6.68-6.8 (at 24 hours)
+: Increase in turbidity
Each tube contains 10.0 ml of liquid medium. 12-hour culture was the inoculumn

TABLE IV

ASSAY FOR TOXIN PRODUCTION BY E. RHUSIOPATHIAE IN VARIOUS LIQUID MEDIA

Exp. No.	Strains of Organism	Medium	Duration of Incubation	Volume* Injected	Route	Dead Mice
1	273	a	44 hours	0.5 ml	I. V.	0/3
2	5483	a	"	0.5 ml	I. V.	0/3
3	5483	a	"	1.0 ml	I. P.	0/3
4	5483	b	48 hours	0.5 ml	I. V.	0/3
5	273	b	"	0.5 ml	I. V.	0/3
6	5483	b	7 days	0.5 ml**	I. P.	0/3
7	273	b	"	0.5 ml	I. V.	0/3
8	5483	c	44 hours	0.5 ml	I. V.	0/3
9	273	c	"	0.5 ml	I. V.	0/3
10	5483	d	48 hours	0.5 ml	I. V.	0/3
11	273	d	"	0.5 ml	I. V.	0/3
12	5483	a	44 hours	0.5 ml	I. M.	0/3
13	273	a	"	0.5 ml	I. M.	0/3
14	273	a	"	0.5 ml**	I. V.***	0/3
15	5483	a	"	0.5 ml**	I. V.***	0/3

Abbreviations: I. V.: Intravenously, I. P.: Intraperitoneally
I. M.: Intramuscularly

* : Ten-fold concentrated sterile liquid culture

** : Sterile liquid culture

*** : Second injection of sterile liquid culture was administered 2 hours after first injection

a : BHI broth supplimented with 5%(v/v) horse serum and 1%(w/v) yeast autolysate (Pfize)

b : Casitone broth containing 0.05%(w/v) cystine and 0.05%(w/v) sodium thioglycolate

c : Casitone broth containing 0.05%(w/v) tryptophan and 0.05%(w/v) sodium thioglycolate

d : Proteose Pepton No. 3 containing 0.05%(w/v) cystine and 0.05%(w/v) sodium thioglycolate

All incubation were at 37° C in stationary culture aerobically

One hundred ml of brain heart infusion broth containing 5 per cent (v/v) horse serum and 1 per cent (w/v) yeast autolysate (Pfizer) was inoculated with a full loop of stock culture of E. rhusiopathiae strain 5483. After 44 hours, the liquid culture was sterilized by a combination of centrifugation and filtration and then freeze-dried. The freeze-dried sample was reconstituted with 10 ml 0.02 M NH_4HCO_3 and applied to the G-100 column. The chromatography procedures were performed at room temperature and the elution pattern is presented in Figure 1. The dark horizontal bars under the eluted peaks indicate pooled fractions. Each pooled fraction was freeze-fried and reconstituted in 10 ml phosphate buffered saline (pH 7.2) and the toxicity was tested. The remainder of the test sample was kept at -70° C for future use. All three mice injected with the sample from the large molecular weight fraction (peak 1) died about 45 minutes following injection. Tissues from liver and spleen, as well as blood, were cultured by streaking onto blood agar plates. No bacterial colony was isolated indicating that probably no bacterial infection was involved although media enriched for isolation was not used. Peracinar-like necrosis of the liver was observed in each dead mouse. This lesion could be caused by a toxin. However, there is a possibility that the death was due to anaphylatoid shock since precipitation was found in the sample injected. The remainder of the sample, which had been kept at -70°C, was warmed to room temperature and centrifuged at 27,000 xg for 20 minutes in a Servall Superspeed RC 2-B Centrifuge to remove the precipitate. Toxicity of the supernatant was tested again by injecting two mice intravenously with 0.5 ml supernatant. One of the mice died after about 22 hours. No bacteria were isolated from liver, spleen

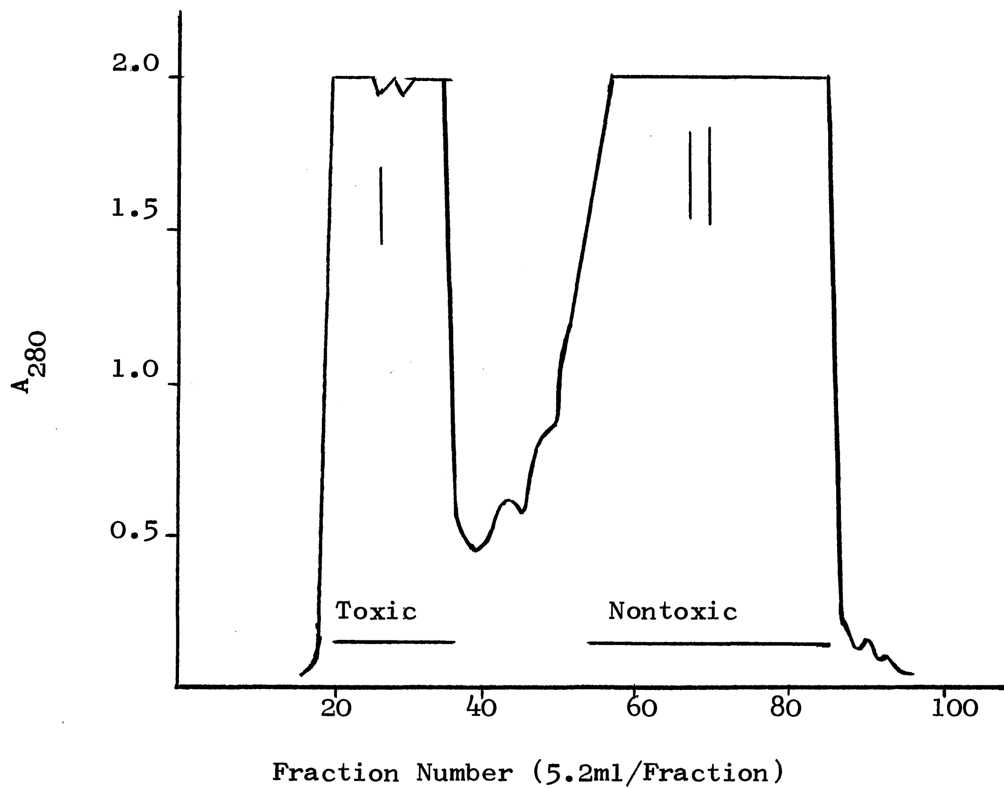


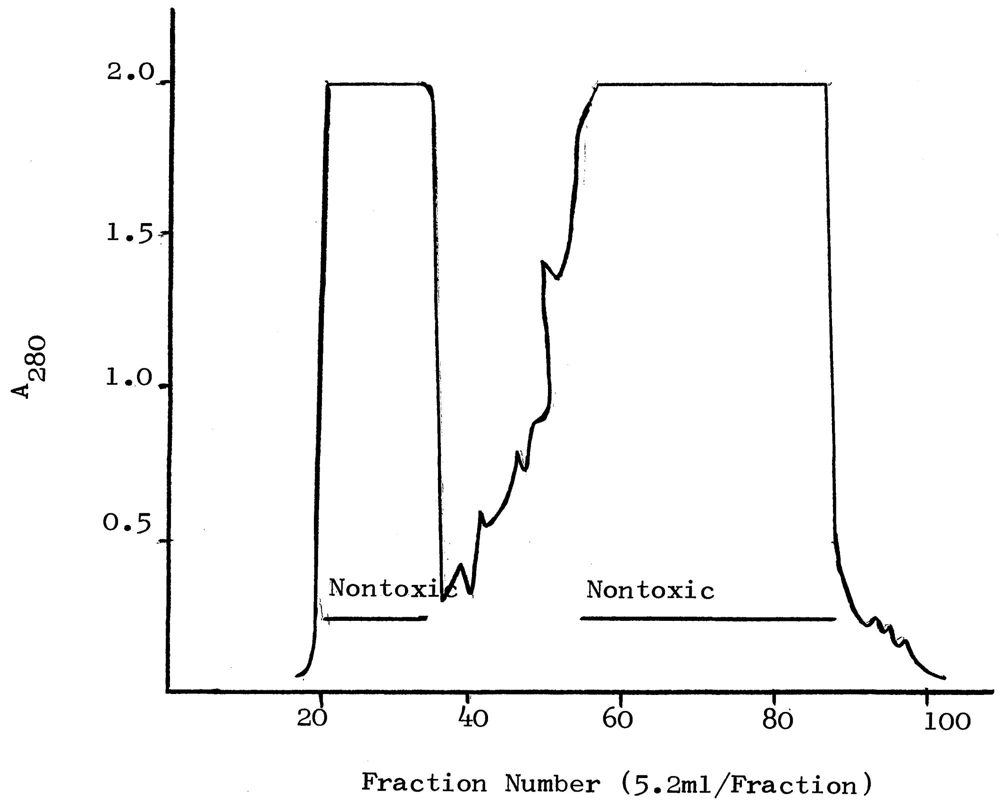
Figure 1. Analysis by Gel Filtration of BH1 Broth *E. rhusiopathiae* Culture for the Presence of a Toxin on Sephadex G-100 Column at Room Temperature

or blood of the dead mouse. Yellow streaks on the surface of the liver were observed in the dead mouse. The rest of the supernatant was kept at -70°C and the toxic activity again tested by a third intravenously injection of two mice. One of the two mice died about three hours after injection. Again, no bacteria were isolated from the liver, spleen and blood of the dead mice.

In the control experiment, the same experiment was performed with exception that fresh medium was used. The eluted pattern is presented in Figure 2. Precipitated material was also found in peak I from Figure 2. All three mice died within five minutes of the first injection of the large molecular weight fraction (Peak I from Figure 2). It seems apparent that the mice died from anaphylatoid shock since typical symptoms of anaphylatoid shock were found (the hair on the back of the mice stood up, they sneezed and died rapidly). The anaphylatoid shock was probably due to the precipitate in the sample, since no mice died from the second or third injections of the samples in which the precipitate had been spun down.

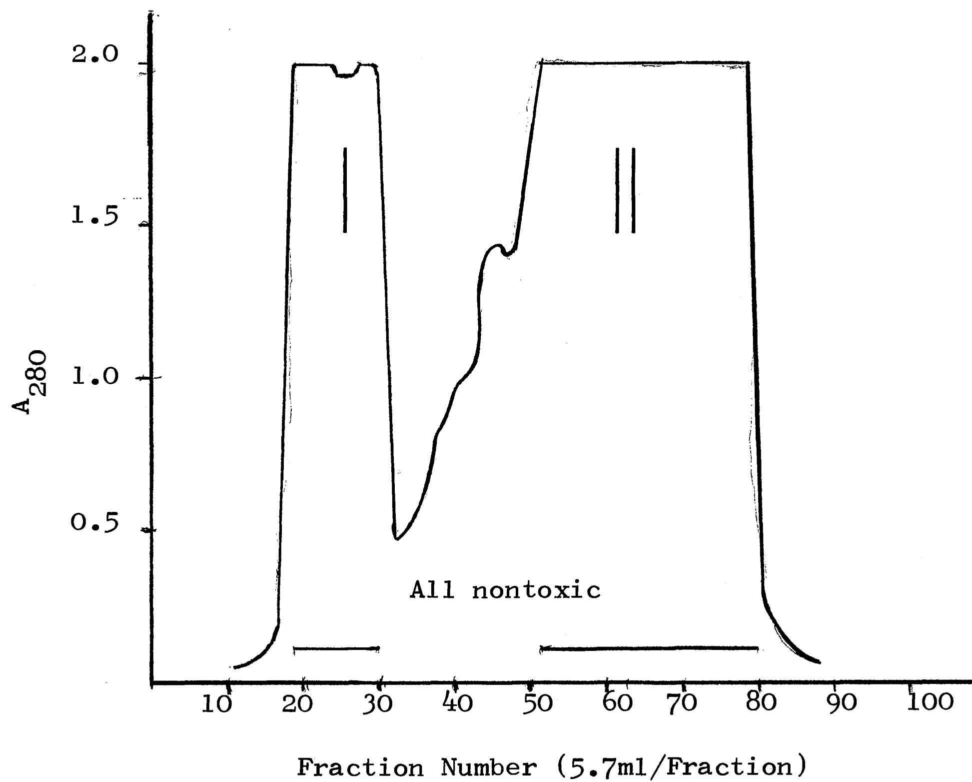
It was apparent from the poor separation that was obtained from these two runs that the column was overloaded. However, large molecular weight substances had been separated from small molecular weight substances and found to be toxic to mice. Therefore, no further separation was considered necessary. It was unnecessary to decrease the total amount of applied sample to increase resolution.

The previously described two fractions were repeated in the cold room at 4°C. The elution pattern is presented in Figures 2 and 4. The pattern of Figure 3 is similar to Figure 1 and that of Figure 4 is similar to Figure 2.



[Conditions were the same as for Figure 1]

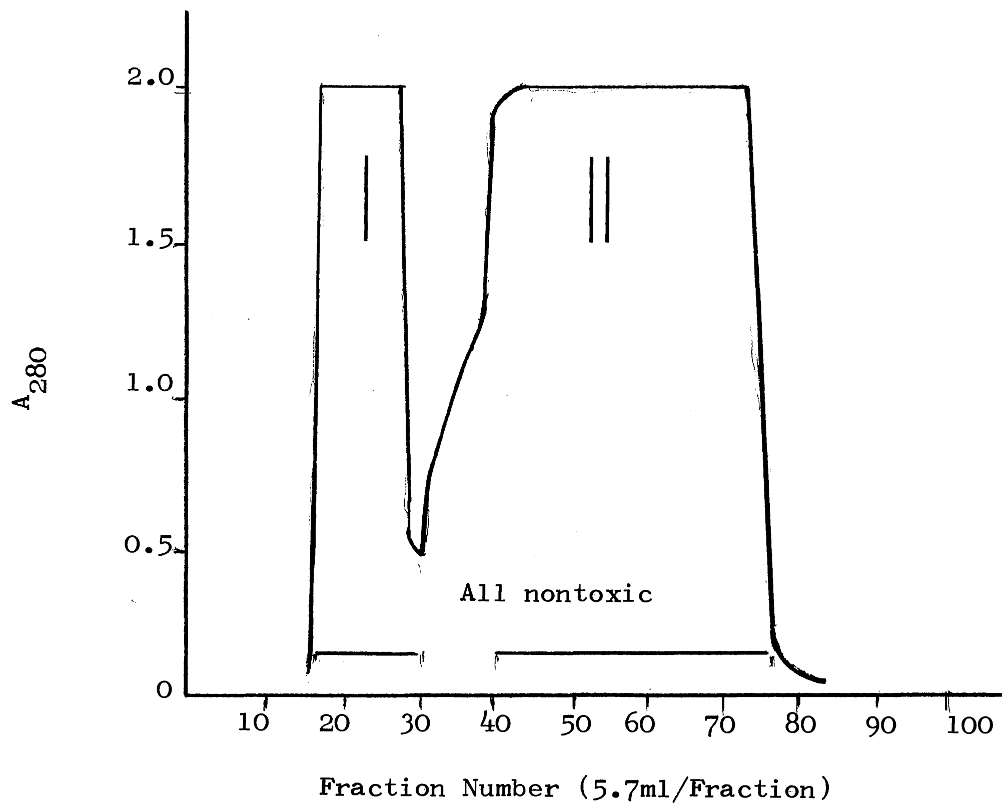
Figure 2. Gel Filtration of Noninoculated BH1 Broth on Sephadex G-100 Column



Fraction Number (5.7ml/Fraction)

[Culture was sterilized after growth of the organism (44 hours). Conditions were the same as for Figure 1 with exception that the experiments were performed at 4°C.]

Figure 3. Analysis by Gel Filtration of BHI Broth <u>E. rhusiopathiae</u> Culture for the Presence of a Toxin



[Conditions were the same as for Figure 2 with the exception that the experiments were performed at 4°C.]

Figure 4. Gel Filtration of Noninoculated BH1 Broth on Sephadex G-100 Column

Toxicity of the pooled fractions (Figures 3 and 4) was tested at the same time by injection of five mice weighing from 25-30 g with 0.5 ml 10-fold concentrated sample. No toxicity was found in any of the fractions obtained. It is interesting to note that larger molecular weight peak I of Figure 3 obtained at 4°C showed a complete loss of toxicity. Further separation of the low molecular weight fraction (peak II of Figure 3) in a sephadex G-25 column resulted in at least six components. Each component was tested for toxicity by injecting I.V. into five mice weighing 25-30 g. No elution pattern is presented here because no toxicity was found.

Discussion

For the assay and purification of a toxin, it is desirable to select the simplest synthetic medium available. Since a satisfactory synthetic medium for growing this organism was not available, nine different types of media were prepared and investigated (see Materials and Methods). The attempts to find a synthetic medium for the organism were unsuccessful. The media tested included a tissue culture medium which is a rather complex defined medium containing most of the growth factors required for growing bacteria. If the organism cannot grow in this medium, it would be hard to grow on any defined medium. Therefore, the search for a defined medium for toxin production was abandoned when it was found that the *E. rhusiopathiae* could not grow in this medium. A decision was made to use proteose peptone No. 3 and casitone. These two media are frequently used as the basic nutrients in bacterial toxin studies.

Tables II and III showed the titer of bacteria from 24 to 64 hour cultures in media using proteose peptone No. 3 and casitone as the basic nutrients which were supplemented with cystine, sodium thioglycolate, tryptophan and yeast autolysate in various combinations. Since the organism showed some growth in the media presented in Table II and Table III, but none in tissue culture medium, this implies that something in peptone is necessary for the organism's growth that is not in tissue culture medium. This material might be a certain kind of peptide.

Results of the inoculation of _E. rhusiopathiae_ into casitone containing medium were determined by titering the bacteria at 34 and 64 hours and checking the macroscopically visible change in turbidity at 24 hours post inoculation. Titers of the culture at 34 hours were found to be almost equal to the initial titers. There was no significant difference between titers at 0 hour and at 24 hours. The almost equal number of bacteria at 0 and 24 hours could be due to maintenance of viability for as long as 34 hours in these media or to a balance between cells produced and cells dying. However, the slight increase in visible turbidity suggested that _E. rhusiopathiae_ did grow, and probably multiplied, to a certain extent. Additional cystine and tryptophan did not shown any significant advantages for the organism's growth.

In order to produce enough exotoxin, it is desirable to have a suitable medium in which the organism can grown abundantly. It is much easier to demonstrate the existence of a bacterial toxin when toxin concentration is high. Abundant growth in liquid medium is necessary for high production of exotoxin _in vitro_. However, even

though E. rhusiopathiae did not grow very well in the media containing proteose peptone No. 3 or casitone, they were still used for toxin studies since the filtrate was concentrated ten-fold. No toxin was demonstrated using mice as an indicator animal under varying growth conditions and injection routes. The results are presented in Table IV. Possible reasons for these negative results are:

(1) Concentration of the toxin produced by the organism might not be great enough. Bacterial growth was not very good in these two media as shown in Table II and Table III. Low metabolic activity might not produce enough toxin to kill the mice in the amount of 0.5 ml of a 10-fold concentrated sample.

(2) There is the possibility that the media lack the special materials which can stimulate toxin production. Pseudomonas aeruginosa, for example, produces lecithinase on tryptone agar enriched with one per cent glucose but no lecithinase is produced without glucose (Liu, 1964).

(3) Neither casitone nor proteose peptone No. 3 supplemented with cystine, sodium thioglycolate, and yeast autolysate are good media for toxin production.

(4) Mice might be a poor assay animal. Although mice are susceptible to E. rhusiopathiae, it is still unknown if mice would be susceptible only to a toxin produced by the organism.

(5) If the toxin produced by the organism is an exotoxin, there is a possibility that the toxin was digested by the proteolytic enzymes which were produced by the organism in the media, since mot exotoxins are proteins.

(6) Toxin may be tied up by a substance in the medium.

A natural medium, BH1 broth supplemented with five per cent horse serum and one per cent yeast autolysate, was also used in these studies. E. rhusiopathiae grew abundantly in this medium but no toxin could be demonstrated in the filtrate (Table IV). There is the possible reason, as discussed above, that the medium might not have been suitable for toxin production. Efficiency of the production of toxin varies with different media used. Since this is a rather complex medium and is supported with horse serum, it has to be considered that the toxin produced might have combined with other components in the medium and been inactivated.

Gel filtration was employed to separate the mixed components in the liquid culture. Attempts were made to find out if the toxicity was depressed by the other components in liquid culture. In the gel filtration study, performed at room temperature, the toxicity which was found in the larger molecular weight fraction of a BH1 broth culture incubated for 44 hours was considered significant. The result suggested that the toxin is a large molecular weight substance and that further purification of the complex sample would be helpful for the demonstration of the toxin.

The column chromatography technique described above allows a small amount of contamination. The presence of contamination would have no effect on the result of the experiment. In several of my previous experiments, growth from contamination in several fractions of the larger molecular weight peak (peak I of Figure 1, 2, 3 and 4) was observed when the experiments were performed at room temperature and the collected fractions were left at that temperature over night.

However, the growth from the contamination was prevented by maintaining the collected fractions at 4°C immediately after collection. No growth from contamination was found in any fractions of the lower molecular weight peak. This might be due to the removal of the growth factor by gel filtration. In the experiment described above, fractions were collected at room temperature and kept in a cold room after they were collected.

However, the toxicity was lost when the experiment was repeated at 4°C. The reason that the toxicity was lost is still unknown. It could be due to unstable production of the toxin in BHl broth. If so, the unstable production of toxin could be due to the unsatisfactory nature of the medium or to the lack of certain materials to stabilize the toxin produced. However, there is a possibility that the cold temperature denatured the toxin. Therefore, for the further study of *in vitro* production of *E. rhusiopathiae* toxin, it will be necessary to find a suitable medium which can stabilize the production of *E. rhusiopathiae* toxin.

Toxicity of lower molecular weight fractions (Fraction II of Figures 1 and 3 have been studied in detail. No toxicity was found, which increases the possibility that the toxin might be a large molecular weight protein.

Mice were killed once by the higher molecular weight fraction of the BHl liquid medium culture, and are, therefore, possibly acceptable animals. However, further studies to definitely determine that mice can serve as assay animals are necessary. The discovery of a good assay system is a primary requirement for further *E. rhusiopathiae* toxin studies. White et al. (1970) reported that a large protective

antigen containing particle or aggregate found in culture supernatant fluid of E. rhusiopathiae was isolated. The physical and biological characteristics of the protective antigen containing particle have been further studied by the same author, and he suggested that the protective antigen is a glyco-lipoprotein (White et al.). Miller (1974) reported that the neuraminidase in E. rhusiopathiae could be an important pathogenic factor in E. rhusiopathiae infection. Blocking this enzyme with antiserum effects the loss of pathogenicity of E. rhusiopathiae. The column chromatography studies presented here strongly suggests that E. rhusiopathiae produced a large molecular weight exotoxin. However, since no toxic material was obtained in repeated chromatography experiments, attempts at further study of chemical and biological characteristics of the toxin were not possible.

CHAPTER III

STUDIES ON THE RELATIONSHIP BETWEEN THE VIRULENCE OF E. RHUSIOPATHIAE AND THEIR PROPHAGE

Materials and Methods

Bacteria

E. rhusiopathiae, virulent strains 1794, 5483, 3162, 1400, 273, 5188 and avirulent strains 150 and AVR-9 were used in this study. All virulent strains were from our laboratory stock and were grown in the yolk sac of embryonal chicken eggs. The original organisms of strains 1794, 3162, 5483 and 1400 were isolated from infected turkeys and had one common ancestor strain, 273. Only virulent 212 was isolated from swine. Avirulent strains 15C and AVR-9 were obtained from Dr. R. L. Wood, National Animal Disease Laboratory, and the stock cultures were maintained on blood agar plates by periodic transfers and stored at 4°C after growth.

Media

Brain heart infusion broth (BHl broth) (Difco) and heart infusion broth (HI broth) (Difco) were used. For the preparation of these media, the manufacturer's suggestions were followed.

For double agar layer plates, the bottom layer of the plates contained 35 ml of HI broth with 1.1 per cent Difco agar and 10 per cent

horse serum, and the top layer contained 0.7 per cent Difco agar and 5 per cent horse serum.

For liquid bacterial culture medium, BHl broth containing 5 per cent horse serum and 1 per cent yeast autolysate (Pfizer) was used.

Chemicals

Mitomycin C was purchased from Sigma Chemical Company. Chemicals from J. T. Baker were calcium chloride ($CaCl_2 \cdot 2H_2O$) and magnesium sulfate ($MgSO_4 \cdot 7H_2O$). Acridine orange was used for the attempt to cure the lysogenic bacteria from their prophage and was purchased from Fisher Scientific.

Selection of the Most Ultraviolet Light-sensitive Strain

Virulent strains of E. rhusiopathiae to be tested were grown in Bhl broth at 37°C for 12 hours. The cells were harvested by centrifugation at 9,000 x g for 20 minutes and washed with 0.01 M phosphate buffered saline (pH 7.2) three times. The turbidity of the tubes of washed cells of each strain was adjusted to the same opacity by visual comparison with the naked eye. Ten ml of the cell suspension of each strain were exposed to ultraviolet light at a distance of 45 cm from the germicidal lamp (type A). Aliquots of 0.1 ml sample was taken from the sample after exposing to UV light at 30, 45, 60 and 90 seconds and was then transferred to 10 ml of BHl broth enriched with 10 per cent (v/v) horse serum and one per cent (w/v) yeast autolysate and then incubated statically at 37° C for 12 hours. Any increase in turbidity as measured macroscopically was recorded. Sensitivity to

the UV light was determined by the amount of time required to show an increase in turbidity.

Demonstration of Bacteriophage by Induction of Cell Lysis

Mitomycin C and ultraviolet light were used as inducers of cell lysis in all experiments of this study.

1. Induction by Mitomycin C. Two different mitomycin C treatments were performed to induce the lysis of virulent strains of *E. rhusiopathiae*.

Treatment A: Two mg of the antibiotic mitomycin C crystals were reconstituted in five ml distilled water. A solution containing 400 µg per ml was used as a stock solution. Five ml BHl broth in tubes containing various concentrations of mitomycin C were prepared by the addition of various amounts of mitomycin C stock solution. Each tube was inoculated with 10^7 - 10^8 organisms and incubated statically at 37°C. The tubes were observed for turbidity changes every 30 minutes for 12 hours by comparison with a control tube. The turbidity control tube was prepared by inoculating the same amount of washed bacteria into a 5 ml BHl broth tube and keeping it at 4°C to slow down bacterial multiplication. A decrease in turbidity indicated that cell lysis had been induced by their bacteriophage.

Treatment B: The preparation of BHl broth tubes containing various concentrations of mitomycin C was the same as described above. A titer of 10^7 - 10^8/ml of the organism to be tested was exposed to the media for 30 minutes. The bacterial cells were then harvested by centrifugation at 9,000 x g for 20 minutes and resuspended in five ml

of BHl broth enriched with 5 per cent (v/v) horse serum. Turbidity control tube was also prepared and kept in a 4°C cold room. Cultures were observed for turbidity changes at 30 minute intervals for 12 hours by comparison with the control tube.

2. Induction by Ultraviolet Light. Washed bacteria were prepared by growing bacteria in BHl broth, harvesting by centrifugation at 9,000 x g for 20 minutes and washing with 0.01 M phosphate buffered saline (pH 7.2) three times. A 10 ml of 0.01 M phosphate buffered solution containing 10^{6} -10^{7} cells per ml was irradiated by ultraviolet light for 30, 45, 60 and 90 seconds at a distance of 45 cm under a germicidal lamp (type A). The irradiated cells were then spun down by centrifugation at 27,000 x g for 20 minutes and resuspended in 5 ml of fresh BHl broth enriched with 10 per cent (v/v) horse serum and one per cent (w/v) yeast autolysate (Pfizer). Each tube was then incubated statically at 37°C. A turbidity control tube was also set up by inoculating the same amount of washed bacteria into a 5 ml BHl broth tube and keeping it at 4°C to slow down bacterial multiplication.

Isolation of Phage-Susceptible Organisms by a Curing Method

A total of 10^{6} -10^{7} washed cells of strain 5483 was inoculated into 10 ml BHl broth containing 5 per cent (v/v) horse serum, 1 per cent (w/v) yeast autolysate (Pfizer), and 2,000 μg acridine orange. The culture was incubated statically at 37°C for 24 hours. Surviving bacteria were isolated by streaking a loopful of culture suspension onto blood agar plates. Each colony isolated was marked AO-1, AO-2 . . . AO-9, and maintained on blood agar plates by periodic transfers

at room temperature.

Isolation of Bacteriophage

Tha agar plate technique, as described by Swanstrom and Adams (1951), was employed for isolating phage from virulent strains of *E. rhusiopathiae*.

A modification of this method, as described below, was also used. An amount of one inoculation loop of filtrate from each treatment was plated onto 3 ml of soft agar layer in which 10^7 - 10^8 seed cells had been added, mixed and poured over 30 ml of the bottom layer which had been poured into a petri dish and kept at room temperature for at least two days in order to evaporate surface moisture. The plates were laid in a horizontal position for 30 minutes to allow the sample to be absorbed by the agar. The plates were then incubated at 37°C in a candle jar.

Preparation of Filtrate for Phage Stock

In order to test for the release of the bacteriophage from the virulent strain *E. rhusiopathiae* into the liquid culture medium, the sterilized filtrate of liquid culture was used as phage stock.

Two methods were used:

A. Tubes containing 10 ml BH1 broth enriched with 5 per cent horse serum were inoculated with a loopful of stock culture of virulent strains and incubated statically at 37°C for 24 hours. After centrifugation at 9,000 x g for 20 minutes, each culture supernate was filtered through a 0.2 μ membrane filter (Gelman Instrument Company).

B. A total amount of 10^5 - 10^6 washed cells of strains 5483, 273 and 1400 in 10 ml phosphate buffered saline (pH 7.2) was irradiated with ultraviolet light for 30 seconds as described above to induce the release of bacteriophage. An amount of 0.1 ml irradiated cells was transferred to 5 ml of fresh BH1 broth enriched with 10 per cent (v/v) horse serum and incubated at 37°C for six hours. A sterile filtrate was obtained by filtering the broth through a 0.2 µ membrane filter (Gelman Instrument Company).

Preparation of Seed Cells

Avirulent strains AVR-9, 15C and the isolates from the medium containing acridine orange were used as seed in the agar plate method for demonstration of the bacteriophage. Seed cells were prepared by propagation in BH1 broth at 37°C for 12 hours and harvested by centrifugation at 9,000 x g for 20 minutes. The harvested cells were washed three times with 0.01 M phosphate buffered saline pH 7.2.

Bacteria Titer

Titers of bacterial cell suspensions were measured by titration as described in materials and methods section of Chapter I.

Results and Discussion

Selection of the Most UV Sensitive Strain

It was suspected that the strain most sensitive to UV light had a higher possibility of harboring a prophage. The purpose of this experiment was to select the most sensitive strain for bacteriophage isolation. The results are presented in Table V.

TABLE V

RESULT OF SELECTION OF THE MOST UV SENSITIVE *E. RHUSIOPATHIAE* STRAIN

Dosage	Bacterial Strains				
	1974	5188	1400	273	3162
30	+	+	±	−	+
45	+	+	−	−	−
60	+	−	−	−	−
90	−	−	−	−	−
Control	+	+	+	+	+

+: Increase in turbidity by naked eye observation

−: No change in turbidity

±: Slightly increase in turbidity

Turbidity was observed by naked eye 12 hours after exposure to UV light

Control: Without exposure to UV light

Strain 273 was determined to be the most sensitive to the UV irradiation since it did not grow within 12 hours after exposure to UV light for 30 seconds, while all other strains did grow. Strain 1400 was determined to be the next most sensitive since cultures showed heavy growth at 12 hours. All the irradiated strains showed an increase in turbidity after 24 hours. Strains 273 and 1400 were thought to have a higher possibility of harboring a prophage and were used for the demonstration of the existence of a prophage by agar plates after the induction of UV, although other strains were also used.

Demonstration of Bacteriophage by Induction of Cell Lysis

Ultraviolet light (Lowff et al., 1950) and mitomycin C (Huang et al., 1970; Prescott and Altonbern, 1967; Levine, 1961) have been shown to induce phage production in a variety of lysogenic bacteria. Both ultraviolet light and mitomycin C have been used for induction of lysis of lysogenic E. rhusiopathiae (Politynska, 1967, 1969). If lysis of the bacterial cells could be induced by UV light or mitomycin C, the bacteria should be lysogenic, and further study of the relationship between virulence and lysogenicity could be pursued.

For the irradiation by ultraviolet light, three virulent strains of E. rhusiopathiae were used. They were 273, 5483 and 1400. No cell lysis was found after treatment with UV as described a ove.

For the induction of cell lysis by treatment with mitomycin C, only strains 5483 and 273 were used. Tubes containing 0.1, 1, 2, 5, 10, 20, 40 and 80 μg per ml mitomycin C were tested. Bacteria were exposed to mitomycin C throughout the incubation period (12 hours).

No decrease in turbidity was observed at any concentration of mitomycin C in comparison with the control tube. It was suspected that the organisms were killed by the antibiotic after such a long exposure. The same experiment, with the exception that strain 5483 and 273 were exposed to mitomycin C only 30 minutes instead of throughout the incubation period, were performed. Again, no decrease in turbidity was found. The results indicated that lysis of our virulent laboratory stock strains of E. rhusiopathiae could not be induced by mitomycin C.

UV and mitomycin C have been used as a standard method for inducing lysis of lysogenic E. rhusiopathiae (Politynska 1963, 1967). From the experimental results obtained, it appears that virulent strains 5483, 273, and 1400 are non-lysogenic. It has been concluded from these results that virulence has no relation to lysogenicity.

Isolation of Bacteriophage from
Virulent E. rhusiopathiae Strains

Lysogenic bacterial strains are not lysed in mass by their phages. They sporadically liberate some phages into the medium due to occasional lysis of some cells. The liberated phage can be detected by the double agar layer plate technique by using a sensitive strain as an indicator. Since an E. rhusiopathiae bacteriophage has been found (Brill and Politynska, 1901) it was suspected that our laboratory stock of virulent E. rhusiopathiae strains might also be lysogenic. It was considered that prophages in the bacteria might be related to virulence, that is, only the virulent strains might harbor the prophage. It this is so, then avirulent strains should be nonlysogenic. Strains sensitive to an E. rhusiopathiae bacteriophage possibly can be

selected from avirulent strains or any other lysogenic strains cured of their prophage. Although no cell lysis was induced in virulent strains 237, 5483 and 1400 by UV and mitomycin C, the filtrate from their liquid cultures which might contain the phages sporadically released into the medium, were worth testing. Avirulent strains 15C and AVR-9 and the 10 isolates from the virulent strain 5483 which were suspected to be cured of their prophage were used as seed cells. Filtrates of all virulent cultures were tested with each of the 11 possibly sensitive strains. If plaques had been found, it would have indicated that the virulent strains had released their phages into the liquid culture and that seed cells were sensitive to them. However, no plaques were found from any plated sample.

Metal ions may be necessary for the phage to attach to the cell surface to enable the phage DNA to enter sensitive bacterial cells. The experiment was repeated with the exception that a concentration of 10^{-3}M Ca^{++} or Mg^{++} was added to the soft agar, and only filtrates of virulent strains 5483, 5188, 273 and 212 were tested. Again, no plaques were observed. Since all strains isolated from turkeys were from the same ancestor-strain, 273, it was considered unnecessary to test all of the strains isolated from turkeys when the experiment was repeated. The only strain isolated from swine used in the repeated experiments was strain 212. Additional Na^+ was considered unnecessary since HI medium contains 0.08 M Na^+.

Another agar plate experiment was done by plating filtrate from strains 273 and 1400 which had been exposed to ultraviolet light for 20 seconds as described above. The filtrate was obtained from a six-hour culture. The purpose of this experiment was to activate the

prophage by UV light irradiation. A higher titer of bacteriophage might be released into liquid media after UV treatment, which would be easier to detect. However, this attempt was also a failure.

The fact that no phage was found supported the conclusion that the strains tested harbor no prophage. However, since the phage sensitivity of AVR-9, 15C and isolates from acridine-treated virulent strain 5483 is still unknown, it is not possible to reach a final conclusion from this experiment. The released phage may be quickly absorbed by lysogenic cells and not be free to interact with susceptable strains, even if AVR-9, 15C and isolates from acridine orange treated samples are susceptable. Also, the interval after treatment with UV or mitomycine C might have been too long. All phage may have been reabsorbed.

CHAPTER IV

ISOLATION OF L-PHASE E. RHUSIOPATHIAE FROM INFECTED TURKEYS

Bacteria

Virulent strain 5219 of *E. rhusiopathiae* was used throughout this investigation. A stock culture was maintained in embryonal chicken egg yolk and stored at -70°C.

Experimental Turkeys

Eight-week old turkeys were obtained from the Oklahoma State University poultry farm. They were raised in houses on litter (sugar cane pumice) until moved to the experimental units where they were kept in outside screened pens on gravel. Six turkeys were inoculated in the upper neck region with 0.3 ml trypticase soy broth in which 1.5×10^7 cells of the organism which had been grown in the yolk sacs of embryonal chicken eggs. When one of the infected turkeys died at 46 hours post-inoculation, the surviving turkeys were given intramuscularly 80,000 units of erythromycin at 46, 70, 94 and 118 hours post-inoculation. The erythromycin was a production of Diamond Laboratories and stored in the refrigerator until used in the turkeys.

Bacteriological Technique

For the isolation of L-form and parent-form of *E. rhusiopathiae* in the infected turkeys' blood, blood was drawn from the large vein on the underside of the wing of the turkeys by using a 3 ml syringe containing heparin and immediately placed in sterile tubes partially immersed in an ice bath. Starting at 46 hours post-exposure, the turkeys were bled at 24-hour intervals through 118 hours post-exposure, then at twice per week through 560 hours post-inoculation. The number of bacteria in the blood was titrated by making 10-fold dilutions of the blood using trypticase soy broth as the diluent. Six 0.01 ml aliquots of each 10-fold dilution were placed on BH1 plates enriched with five per cent bovine blood. The plates were then incubated at 37°C in a candle jar. A 0.5 ml aliquot of each undiluted blood sample was also inoculated into an enriched liquid medium (Wood, 1965) which was incubated at 37°C for three days and then streaked onto blood agar plates enriched with five per cent horse serum and containing crystal violet and sodium azide as recommended by Packer (1943). The plates were incubated at 37°C in a candle jar for three days. Isolated colonies typical of *E. rhusiopathiae* were transferred to triple sugar iron medium to determine characteristic reactions (Vickers and Bierer, 1958). Confirmatory tests were done by using a plate agglutination test with the isolated organism as the antigen and *E. rhusiopathiae* chicken antiserum.

Plate Agglutination Test

A plate was warmed to 37°C in an incubator and marked with six circles. An amount of 0.08, 0.04, 0.02, 0.01 ml and 0.005 ml of chicken anti-erysipelothrix-antiserum was pipetted on succeeding rings of the agglutination plate. A drop of 0.03 ml E. rhusiopathiae suspension, which was equivalent to McFarland tube #6, was added to each drop of serum. The 6th circle contained only antigen. The plate was examined for agglutination after a few minutes of tilting the plate gently back and forth.

Antiserum

Virulent strain 273 was grown in BH1 broth enriched with 10 per cent horse serum and one per cent yeast autolysate, incubated at 37°C for 24 hours and harvested by centrifugation at 9,000 x g for 15 minutes. The cells were then resuspended in 0.3 per cent formalized saline and kept at 4°C overnight to kill the bacteria. Sterility was checked by transferring 0.1 ml suspension to 40 ml trypticase soy broth which was then kept at 37°C for three days. The killed bacterial cells were then diluted with 0.01M phosphage buffered saline (pH 7.2) to contain approximately 5.2×10^9 cells of the organism per ml. An equal amount of the killed antigen and complete Freund's adjuvant (BBL) was mixed in a sterile 10cc flask with a syringe. After mixing well, one ml of the mixture was injected subcutaneously into the upper neck of the turkeys. The second injection of the turkeys was with a live vaccine with complete Freund's adjuvant three weeks later. The preparation of the live vaccine was done the same

way as for the killed vaccine with the exception that washed bacterial cells were suspended in 0.01 M phosphate buffered saline that contained no formalin. Three weeks after the second injection, the turkeys were killed and serum was collected and kept at -70°C.

Media

For bacterial cell propagation, brain heart infusion broth enriched with 10 per cent horse serum and one per cent yeast autolysate was employed.

For the isolation of L-form bacteria from infected turkeys, PPLO medium (Pfizer) enriched with 10 per cent (v/v) horse serum and one per cent (w/v) yeast autolysate was used. PPLO agar plates contained two per cent agar (Difco) while the PPLO semisolid medium contained 0.4 per cent agar (Difco). Preparation of the stock PPLO medium followed the manufacturer's suggestions. All the PPLO inhibited medium was prepared by adding 1,000 units potassium penicillin G and 0.25 mg thallium acetate per ml of medium. Potassium penicillin G was purchased from Pfizer Laboratories Division. The stock solution was prepared by following the manufacturer's suggestion. Thallium acetate stock solution was prepared by dissolving 2.5 g of crystals in 100 ml H_2O and autoclaving at 121°C for 10 minutes.

Isolation of L-form of *E. rhusiopathiae*

A 0.1 ml heparinized blood sample from each infected turkey was inoculated into three ml of PPLO inhibited broth containing 10 per cent horse serum, 1,000 units Potassium Penicillium G and 0.25 mg thallium acetate per ml. The tubes were incubated at 37°C aerobically.

Passages were done by transferring 0.5 ml of culture to 5 ml of fresh PPLO inhibited broth after 72 hours incubation. At each passage, 0.05 ml of broth was spread by a glass rod onto a PPLO inhibited agar plate containing the same amounts of inhibitors and horse serum. The plates were then incubated at 37°C in a candle jar in which there was a five inch tube containing water for maintenance of a high moisture level in the jar. The plates were checked for development of L-form colonies each day for 10 days, starting the third day of incubation. Any suspected colony was transferred onto a PPLO inhibited plate to distinguish true colonies from artifacts. The plates were incubated at 37°C for 14 days in a candle jar and examined every day.

A similar experiment was done at the same time by using the same procedures with the exception that PPLO inhibited semisolid medium was used instead of PPLO inhibited broth.

Results and Discussion

The purpose of this experiment was to isolate L-phase *E. rhusiopathiae* from infected turkeys. After infection with virulent strain 5219, the first turkeys in the group died 46 hours after inoculation. Of the surviving turkeys, some showed no clinical symptoms and some were slightly depressed. Erythromycin was given at that time to decrease the number of bacteria in the blood stream to a low level and to protect the turkeys from clinical disease. Examination for change of the parent form to L-form was performed by attempted isolation of the L-form from the blood.

The isolation of *E. rhusiopathiae* from the blood of erythromycin medicated turkeys is presented in Table VI.

TABLE VI

RESULTS OF THE ISOLATION OF L-PHASE AND PARENT FORM *E. RHUSIOPATHIAE* FROM INFECTED TURKEY'S BLOOD

Hours of Inoculation	Bird Number 974		788		961		793		975	
	L	P	L	P	L	P	L	P	L	P
46	0	$2x10^3$	0	$2.8x10^5$	0	$2.8x10^6$	0	$5.6x10^7$	0	$2.8x10^3$
70	0	$2x10^2$	0	$2.0x10^2$	0	25	0	$2.0x10^3$	0	2
94	0	25	0	25	0	$6.2x10^3$	0	25	0	2
118	0	2	0	25	0	25	0	2	0	2
152	0	0	0	2	0	25	0	2	0	2
224	0	25	0	0	0	25	0	$1.0x10^3$	0	25
320	0	2	0	0	0	0	0	$2.5x10^4$	0	25
422	0	2	0	2	0	2	0	2	0	0
488	0	0	0	0	0	0	0	0	0	0
560	0	0	0	0	0	2	0	0	0	0

Turkeys were mediated with 80,000 UG erythromycin at 46, 70, 94 and 118 hours post-inoculation. The parent form is given as the number of organisms/ml of blood.

L: L-phase
P: Parent form
25: One colony was isolated from one loopful of undiluted blood. One loopful of sample was considered to be 0.04 ml aliquot; there would be 25 colonies/ml of blood.
2: Organisms were only isolated by enrished medium. An amount of 0.5 ml blood sample was inoculated into 5 ml of enriched broth. If growth was obtained it was considered to have organisms at least one organism/.05 ml of blood.
0: No organism was isolated.

At 46 hours post-inoculation, a very high number of bacteria, varying from 10^3 - 10^7/ml were isolated. After the first administration of the antibiotic, the number of bacteria in the blood was reduced to a lower level (25-10^3/ml). Bacteria in the blood stream reached an undetectable level after 488 hours post-inoculation except in bird No. 961. Persistence of bacteria in infected turkeys could be isolated at 560 hours post-inoculation from bird No. 961.

In bird No. 975, the number of bacteria remained at 2/ml from 70 hours to 320 hours post-inoculation. No bacteria were isolated after 442 hours post-inoculation. In bird No. 961 the number of bacteria decreased from 1.8 x 10^6/ml at 46 hours to 25/ml at 70 hours post-inoculation and then increased to 6.2 x 10^3/ml at 94 hours post-inoculation. Bacteria remained at 25/ml through 224 hours post-inoculation and decreased to a level of 2/ml at 224 hours. The level of 2/ml of bacteria in the blood stream was found at 442 and 560 hours post-inoculation, although a decrease to an undetectable level was found at 320 and 488 hours post-inoculation. The same fluctuating patterns was also found in all other birds. A similar result has been reported by Corstvet et al. (1970).

During the period of 46-152 hours post-inoculation, a fluctuating pattern was not observed except in the case of bird No. 961. The fluctuating pattern was apparently observed after 152 hours. A possible explanation for this fluctuating phenomenon existing in surviving turkeys is that the reversion between L-phase and parent-form bacteria contributed to this fluctuating pattern. The reduction in the bacterial titer could be due to both phagocytosis and induction of the parent-form to L-form bacteria. The increase in the bacterial

titer could be due to the multiplication of parent-form bacteria which reverted from the L-form. Long existence of L-phase bacteria might play the major roll in long persistence of infection. Therefore, the L-phase of the organism might be able to be isolated during the long persistence period.

Isolation of L-phase _E. rhusiopathiae_ from the blood was attempted 10 times by use of PPLO inhibited broth and semisolid medium as described above. Both potassium penicillin G and thallium acetate were used as inhibitors. Potassium penicillin G was chosen to prevent the L-form in blood from reverting back to the parent-form on an artificial medium. A PPLO inhibited medium containing 20 per cent horse serum was also utilized for the isolation of the L-phase organism. It was also unsuccessful. A PPLO medium containing only potassium penicillin G or thallium acetate was also employed to grow the organism. This did not yield positive results.

Pachas and Currid (1974) reported that the L-form of _E. rhusiopathiae_ could be induced by penicillin and cultivated on nutrient solid agar plates. Furthermore, morphologic entities resembling the L-phase of _E. rhusiopathiae_ have been observed _in vivo_ by fluorescent antibody technique (Corstvet, unpublished data). It is apparent that the L-form organism can not grow on the PPLO medium (Difco) used in these studies. A specific medium other than the PPLO medium must be developed for the isolation of L-phase of _E. rhusiopathiae_ from infected turkeys.

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