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A Study of Salmonella Pullorum and Pullorum Disease in the Chick Employing Infrared Spectroscopy

J. A. Cameron
University of Tennessee-Knoxville

To the Graduate Council:

I am submitting herewith a thesis written by J. A. Cameron entitled "A Study of Salmonella Pullorum and Pullorum Disease in the Chick Employing Infrared Spectroscopy." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Life Sciences.

D.H. Holtman, Major Professor

We have read this thesis and recommend its acceptance:

Accepted for the Council:
Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

July 30, 1958

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W. T. Hallman
Major Professor

We have read this thesis and recommend its acceptance:

J. Orin Nuendt

John M. Woodward

Virden E. Hunt

M. C. Bell

Accepted for the Council:

Dean of the Graduate School

A STUDY OF *SALMONELLA PULLORUM* AND PULLORUM DISEASE IN THE CHICK EMPLOYING INFRARED SPECTROSCOPY

A THESIS

Submitted to
The Graduate Council
of
The University of Tennessee
in
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for the degree of
Doctor of Philosophy

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by

J.A. Cameron

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J. A. C.

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INTRODUCTION

The occurrence of toxic fractions associated with the cell wall of gram negative microorganisms has been investigated and reported by many workers. These reports have been based on results from a limited number of strains of various organisms from which the fractions have been extracted in many different ways. From these reports there appears a mass of information confirming the nature of the fractions as consisting of a complex of lipid, carbohydrate and protein.

Two types of investigation have been overlooked in past work. There have been few reports of the comparison, under identical conditions, of preparations extracted by the various methods from a single species of organism. The second line of investigation concerns the nature of preparations from a number of strains of the same species of organism which differ in virulence. By a comparison of the similarities and differences in the chemical nature of the preparations and a correlation of these properties with the biological property of toxicity, the latter study could yield information regarding the active toxic portions of the endotoxins of gram negative organisms.

In recent years the development of modern spectrophotometric techniques has been of great value in the study of chemistry and of biology and their interrelationships.

The use of spectrophotometric techniques in the visible and ultraviolet regions of the spectrum has reached a high degree of refinement. Only since 1947 has the spectral region of the infrared been used to a significant degree in the study of the biological and biochemical problems. The use of infrared spectroscopy appears to have great potential as a tool in the solution of biological problems. It has been shown to be extremely valuable in identifying inorganic and organic compounds by revealing the presence of specific molecular configurations.

Problems involving the biochemical and physiological interactions of the host-parasite relationship would appear to be an excellent area of endeavor in which infrared spectroscopy could be of great value. There have been sufficient preliminary reports of related problems to justify an attempt to utilize this tool.

This dissertation is concerned with a comparison of a number of endotoxins which have been obtained by one technique from a number of strains of Salmonella pullorum, ranging from a high degree of virulence to complete absence of infectivity.

Infrared spectroscopy has been used to compare the nature of these preparations in an effort to detect possible differences in the chemical nature of the preparation which might account for the differences in virulence of the whole

cells. Before comparing the spectra of the various strains of S. pullorum, the spectra of the whole cells have been compared in order to determine whether or not gross differences in the cells exist.

The degree to which the endotoxin of the organism determines its virulence has been investigated. A quantitative study of the amount of endotoxin yielded from the various strains of S. pullorum by one technique of extraction is reported.

It seems possible that some changes in the chemical nature of the host tissue are elicited by the parasite or by its endotoxin. Major changes of this nature have been sought by the use of infrared spectroscopy.

II. SURVEY OF LITERATURE

The etiological agent of pullorum disease was first isolated by Rettger (1900) in 1899. It was described as a gram negative bacillus found in the liver, intestine, spleen and heart blood of young chicks. The name Bacterium pullorum was proposed for this organism by Rettger (1909). Bergey (1923) has designated the organism Salmonella pullorum. Pullorum disease has been characterized comprehensively in regard to the etiological agent, ecology, epidemiology, mode of transmission and serological testing by Biester and Schwarte (1948).

The occurrence of a toxic substance within the bacterial cell was first postulated by Roux and Yersin in 1888. In 1894 the term endotoxin was first used by Pfeiffer to describe compounds extracted from the typhoid bacillus and from Vibrio cholerae.

Olitsky and Kligler (1920) isolated an endotoxin from the Shigella dysenteriae. This preparation, which was heat stable, was formed in the later period of growth and exerted a typical action on the intestinal tract.

One of the first comprehensive attempts to isolate endotoxins from microorganisms was that of Boivin and Mesrobeanu (1933). Eighteen to twenty-four hour cultures of Bacillus aertrycke were washed and suspended in distilled water and extracted with an equal volume of 0.5 N trichloro-

acetic acid for three hours in the cold. The cells were then removed by centrifugation, and the supernatant dialyzed. The endotoxin was removed from the dialyzed supernatant by precipitation with ethyl alcohol. The lethal dose of such a preparation for 20 g mice was found to be 0.1 mg. The cells were noted to be morphologically intact and to retain their staining characteristics after extraction with trichloroacetic acid. The endotoxin extracted by this method composed approximately five per cent of the dry weight of the cells. The toxicity of the preparation was drastically reduced by hydrolysis with dilute acetic acid. The hydrolysis yielded 20 per cent lipid and 40 to 50 per cent carbohydrate. On the basis of this analysis the endotoxin was thought to be a glucolipid. The remaining 30 per cent of the complex was not identified. The possibility of the presence of a protein moiety was ruled out by the usual qualitative tests available at that time. Similar preparations were obtained from the dysentery bacillus, from Salmonella enteritidis and a number of other gram negative bacteria. The glucolipid was found primarily in the smooth strains of the organisms, and only small amounts were obtained from rough strains.

In the years immediately following these discoveries, Boivin and his associates (1934, 1935, 1938) applied this extraction technique to many different species of bacteria.

In a comparison of these extracts it was found that their chemical properties appeared to be identical, and their toxicity for mice was approximately equal.

During this same period of time Raistrick and Topley (1934) tried to isolate endotoxins with a different technique. A tryptic digest of acetone-killed cells of Salmonella typhimurium was prepared and the endotoxin precipitated by the addition of ethyl alcohol. The toxicity of these preparations was studied by Martin (1934), who found that 0.1 to 0.5 mg was lethal for mice. This preparation represented approximately ten per cent of the dry weight of the bacterial cells. Chemical analysis after hydrolysis with mild acid or alkali demonstrated the presence of a small amount of nitrogenous material as well as a lipid and a carbohydrate fraction.

Ligas (1937 a, b, c) investigated the toxins of Escherichia coli, isolating a number of endotoxic fractions. In using the method of Boivin both polysaccharide and lipid fractions were isolated, and it was concluded that the polysaccharide represents one of the principal constituents of the endotoxin.

Topley et al. (1937), using the extraction technique of tryptic digestion, isolated endotoxins from two strains of Salmonella typhosa. Both preparations contained protein as shown by qualitative tests, but the preparations could

not be precipitated by trichloroacetic acid.

In 1938 Henderson and Morgan extracted endotoxins from the O, W, Vi and rough Vi strains of S. typhosa with diethylene glycol. The endotoxins were precipitated from the extracts with acetone or alcohol. Protein was not demonstrable by qualitative tests. The lethal dose for mice was 0.5 mg for the endotoxin of the rough Vi strain and 0.3 mg for the preparations from the O and W strains.

Endotoxins from S. typhimurium and S. typhosa were extracted by Walker (1940), who used urea and precipitated with 68 per cent alcohol. The endotoxin prepared from S. typhosa was toxic in 0.2 mg amounts for 20 g mice, while 0.4 mg of the preparation of S. typhimurium was toxic for similar animals. Chemical analyses of the preparations revealed polysaccharide and lipid.

The work of Morgan and Partridge (1941) demonstrated the presence of a protein moiety in the endotoxins of S. typhosa and the Shigella dysenteriae. The only prior mention of the presence of protein in preparations of endotoxin was that of Topley et al. (1937), who noted it in preparations from two strains of S. typhosa. Other workers up to this time had repeatedly reported the endotoxins of Salmonellae as glucolipid, protein-free complexes. The presence of protein was again confirmed by Freeman and Anderson (1941), who obtained a preparation of endotoxin from S. typhosa Ty 2

by tryptic digestion followed by extraction with diethylene glycol. Subsequent hydrolysis by mild treatment with acetic acid revealed, upon chemical analysis, that the endotoxin was composed of 50 to 60 per cent polysaccharide, about 16 per cent insoluble polypeptide, 10 to 20 per cent soluble nitrogenous components and 3 to 4 per cent lipid. Later studies by Freeman (1943) with S. typhimurium yielded similar results.

Zahl et al. (1943) extracted endotoxins from Shigella paradysenteriae, S. typhimurium and Rhodospirillum rubrum with two per cent phenol. The preparation was isolated by precipitation with 66 per cent acetone. It was found that immunization of mice with preparations of homologous or heterologous organisms protected the animals against the induction of hemorrhage in implanted tumors. It was felt that these results supported the concept that a common antigenic endotoxin is found among all gram negative bacteria.

Endotoxin preparations were prepared by Boor and Miller (1944) from Neisseria gonorrheae and Neisseria meningitidis by trichloroacetic acid extraction. These preparations did not differ significantly from those obtained from other organisms. No differences were found in the toxicity of equal amounts of endotoxin from various strains of gonococci and meningococci, regardless of bacterial virulence.

Tauber and Garson (1957) have prepared the endotoxin of N. gonorrheae by lysing the cells and precipitating with

acetone. This endotoxin differs from the other reported preparations in that it appears to be primarily a protein with a small amount of glucolipid present.

A highly toxic preparation was obtained from Pasteurella pestis by Baker et al. (1947). This endotoxin was prepared by extracting acetone-dried cells with neutral salt solutions. A water soluble fraction was obtained which had an LD_{50} for 20 g mice of 8 to 15 ug.

Tal and Goebel (1948) prepared the specific antigen of S. paradysenteriae by extraction with 50 per cent pyridine. This product was found to contain 4.5 per cent nitrogen as well as 11 per cent lipid and 1.44 per cent phosphorous. The total yield of antigen was approximately 6 per cent of the dry weight of the cells.

Evidence that all the toxic properties of S. dysenteriae are due to a single endotoxin was presented by Boroff (1949). Boroff examined the toxicity and antigenicity of both whole cells and cellular fractions of the organism. Whole cells of the smooth variant and all its fractions produced identical antibodies which gave cross reactions in any antigen-antibody combination. The rough variant and its derivatives failed to elicit the production of agglutinins and precipitins in rabbits. It was concluded that the somatic antigen and the endotoxin are synonymous.

An antigenic preparation from acetone-dried cells of S. typhosa was extracted with urea by Fuwajima (1952). The

LD_{50} of these preparations ranged from 33 to 36 ug per gram of body weight of experimental animal. Repeated sub-lethal injections of the antigens produced high agglutinin titers in the blood of animals. Such treatment protected the animals against challenges as high as 800 LD_{50}'s.

Watanabe (1952) extracted and fractionated a complete somatic antigen from S. typhosa, strain 58. The extraction was carried out with 0.3 per cent acetic acid, and the antigen was precipitated with alcohol. The preparation was stable when treated with hot acids. Treatment with alkali at 37 C removed the Vi and lipid components. The polysaccharide portions then were separated from each other by hot acid.

Webster et al. (1955) prepared a highly purified somatic antigen from S. typhosa. Initial extraction was carried out with trichloroacetic acid followed by precipitation from a saturated salt solution. Further purification was carried out by precipitation with ammonium sulfate. The preparation contained 60 to 70 per cent polysaccharide, 20 to 30 per cent lipid and 3.5 to 4.5 per cent hexosamine. The purified antigen equalled or exceeded preparations containing 5 to 20 per cent protein in its stimulation of antibody production and pyrogenic activity. This preparation was characterized by infrared spectroscopy and the spectrum compared with that of several other preparations.

Westphal et al. (1952, 1955) and Lüderitz and Westphal (1952) prepared a lipopolysaccharide from Escherichia coli which was toxic and pyrogenic. The extraction was made in a phenol-water solution and the lipopolysaccharide precipitated with alcohol. Analysis of the preparation showed 48.8 per cent carbon, 6.8 per cent hydrogen, 9.97 per cent nitrogen and 2.08 per cent phosphorous. The lipid fraction represented 12 to 13 per cent of the product. Analysis for sugars after hydrolysis showed that the polysaccharide contained d-galactose, mannose, glucose, rhamnose, xylose and N-acetyl hexosamine.

Hydrolysis of the somatic polysaccharide of S. typhosa by Pon and Staub (1952) yielded glucose, galactose, mannose and rhamnose. The polysaccharide was obtained by extraction with diethylene glycol.

Nakagawa (1954) extracted the antigenic polysaccharide of Salmonella newington with trichloroacetic acid. Chromatographic analysis for monosaccharides revealed the presence of mannose, galactose, rhamnose, xylose, glucosamine, N-acetyl glucosamine, a hexuronic acid and an unknown carbohydrate.

Protective antigenic preparations of Pasteurella tularensis were prepared by Ormsbee and Larson (1955). Two procedures were used to obtain antigens. One method consisted of the extraction of cells with ethyl ether and re-

moval of the antigen by precipitation with ammonium sulfate. The other preparation involved extraction in phenol and precipitation with ethyl alcohol. The chemical and physical properties of the preparations were studied and compared. It was suggested that the polysaccharide moiety consists primarily of N acetyl glucopyranoside. Infrared spectroscopy of the antigen revealed a strong similarity to the antigen of S. typhosa as prepared by Webster et al.

It has been suggested by Freter (1956) and by Macpherson (1956) that normal extractions do not liberate all the somatic antigens or toxic products. MacPherson found that trichloroacetic acid failed to extract all the somatic antigens of a number of strains of Escherichia. Freter obtained two different toxic fractions from Vibrio cholerae. It was stated that the methods for the extraction of these fractions did not involve any procedures which could not be potentially realized in the infected host.

Takeda et al. (1954, 1955) have investigated the active factors of the antigens of Shigella flexneri. The pyrogenic and toxic factors were found to be intimately associated. It was assumed that these characteristics of pyrogenicity and toxicity were dependent upon the association of carbohydrate, phospholipid and polypeptide moieties. It was found that the toxic factor does not involve nucleic

acids. The antigen was obtained by extraction with acetic acid.

Sasaki (1956 a, b, c, 1957) has studied the monosaccharide composition of a number of bacteria including: Salmonella anatum, Salmonella senftenberg, Salmonella newport and S. flexneri. The Boivin extraction of these organisms contained, in common, glucose, galactose, mannose, rhamnose, xylose and N-acetyl-glucosamine. A similar preparation of Pasteurella pseudotuberculosis rodentium contained the above compounds except for mannose and contained arabinose.

The lipid moiety of the O antigen of S. flexneri was split be Kasai (1956). Fifty per cent of the lipid portion of a phenol extracted endotoxin may be removed by acid formamide without any change in toxicity. The firmly combined lipids were believed to have a close relationship to the toxicity.

The endotoxin of S. pullorum has received scant attention. In 1915 Smith and TenBroeck found a toxin in culture filtrates of S. pullorum when the cultures were held at 37 C for two days. Intravenous injections of the filtrates into rabbits killed the animals within two hours. The rabbits exhibited symptoms similar to those of anaphylactic shock. Since it was difficult to induce immunity to this toxin in laboratory animals, it was concluded that the toxin

was an endotoxin. The endotoxin was resistant to heating to 60 C for one hour. Boiling for 15 minutes partially reduced the activity of the filtrates.

Hanks and Rettger (1932) sought a specific extracellular toxin of S. pullorum. Three experiments were conducted with cells washed from proteose infusion agar. In the first experiment the cells were suspended in sterile distilled water and heated to 60 C for 40 minutes. Then they were stored for ten days and injected into chicks. The second experiment resembled the first except that the suspending fluid was sterile broth. In the third experiment the cells were treated as in the first work, then the suspension was frozen and thawed 12 times. The material then was filtered and injected into animals.

From the results of these experiments it was concluded that the cells of S. pullorum contain a toxic fraction which is heat resistant and toxic in varying degrees for rabbits, guinea pigs and mice. These preparations caused no noticeable harmful effects when introduced into chicks, however. Because of these findings it was concluded that pullorum disease was a septicemia rather than a toxemia.

S. pullorum was used by Salton and Horne (1951 a) in their studies of the cell wall of bacteria. Cell suspensions were heated to 75 C for five minutes in distilled

water and agitated. At the end of the heating period the flasks were plunged into ice cold water and the cell debris was removed by centrifugation. The cell walls were separated from the supernatant by centrifugation at 10,000 rpm for ten minutes, and the purity of the preparations was verified by obversation with the electron microscope.

The ultraviolet spectra of these cell wall preparations were then studied by Salton and Horne (1951 b). The absence of a peak at 258 to 260 mu indicated that nucleic acids were not present in these preparations. These findings conflict with those of Stacey (1949), who reported nucleic acid components in cell walls. In later studies Salton (1953) found that the polysaccharide portion of the cell wall of S. pullorum consisted of rhamnose, galactose and glucosamine.

The rhamnose content of cell wall fractions was used by Webster et al. (1955) to estimate the polysaccharide present. A number of Salmonella species, including S. pullorum, were used in these studies. The total rhamnose content of S. pullorum was given as 0.74 per cent of the total dry cell weight and 3.8 per cent of the somatic polysaccharide.

Dooley (1955) extracted endotoxin from S. pullorum using two methods of trichloroacetic acid extraction. The more toxic preparation was precipitated from a salt-satu-

rated trichloroacetic acid extract of dried cells. The LD_{50} of the preparation was 4.33 mg for a 35 g chick, while the LD_{50} for the product of the Boivin extraction was 6.04 mg for a 35 g chick. Infrared absorption studies of the two endotoxins indicated that they are similar to other endotoxins or somatic antigens isolated from various gram negative bacteria.

The use of the infrared region of the spectrum in chemical analysis by the use of characteristic frequencies was reported in 1911 by Coblentz. Even at such an early date in the use of infrared spectroscopy, the suggestion was made that the biologist might well approach some of his problems with this tool. Preliminary work of this nature was reported by Stair and Coblentz (1935).

May and Grenell (1957) summarized the work done up to that time in the study of tissues by infrared spectroscopy. The tissues which have been studied include those of plants, insects, skin, osteoid tissues, blood and the vascular system, muscle, nervous tissue and the tissues of the viscera.

Schwartz *et al.* (1951 *a*, *b*) have studied and reported on the infrared spectra of the liver, muscle, heart, kidney, thymus, spleen and other tissues of the rabbit. The spectra of these tissues possess absorption bands at almost identical locations below 8.10 microns. Above 8.10 microns the pattern of absorption was characteristic for each organ,

and the type of tissue could be identified by its spectrum.

An extensive catalog of absorption curves for both normal and neoplastic tissues has been compiled by Woernley (1952). In addition, the spectra of a number of compounds of biological origin and of known purity were recorded and correlated with the spectra of some of the tissues.

The study of bacterial cells by infrared spectroscopy has been undertaken in an effort to provide a rapid method for the identification of bacterial species. The earliest comprehensive work on the infrared spectra of microorganisms was that of Randall et al. (1951, 1952), Smith et al. (1954, 1957) and Kubica et al. (1956), who characterized strains of Mycobacterium by the composition of their lipid extracts. They found that, by maintaining constant conditions, reproducible results could be obtained and certain mycobacterial strains identified.

An investigation of pneumococcal polysaccharides was reported by Levine et al. (1952, 1953 a). The first attempt to identify bacteria by the infrared spectra of the whole cells was that of Stevenson and Bolduan (1952) who demonstrated that many species of bacteria were sufficiently varied in chemical composition to be differentiated by their infrared spectra. This work was continued by Levine et al. (1953 b) who found that the infrared spectra of Aero-

bacter, Escherichia, Salmonella and Shigella species may be differentiated under certain conditions. The effect on the spectrum of variation in the growth medium was demonstrated.

The early work of Stevenson and Bolduan (1952) was critically investigated by Thomas and Greenstreet (1954) who found that the claims of Stevenson and Bolduan were well founded.

Levine et al. (1955) found that spectral typing of Klebsiella by infrared spectrophotometry of crude capsular polysaccharide largely paralleled serological typing. In some cases it was necessary to establish spectral subtypes, while in others different serological types could not be resolved.

A comparison of the infrared spectra of bacterial strains resistant to various antibiotics and to antituberculous compounds and their parent strains was made by Kull and Grimm (1956 a). Qualitative spectral differences could be observed between certain sensitive and resistant strains of Bacillus megaterium and the strain of BCG Mycobacterium tuberculosis variety bovis. These deviations could not be related quantitatively to the degree of resistance. Many resistant mutants failed to show significant difference from parent strains. It was concluded that it is not possible to identify bacterial species unequivocally by means of infrared absorption spectra.

A very extensive description of the work which has been done on the identification of bacterial species by whole cell infrared spectra was that of Riddle et al. (1956). Spectra of 650 strains of 201 species of 33 genera of bacteria were recorded. These spectra were manipulated in order to code them and use this code on IBM cards to form a catalog of spectra.

Greenstreet and Norris (1957) examined the infrared absorption spectra of 137 strains of bacteria. It was found that genera could be distinguished easily, while species could be distinguished with difficulty. Differences between strains of the same species were of the same order of magnitude as those arising from error of technique.

O'Connor et al. (1957) described a method for differentiation of bacteria by comparison of the infrared spectra of aqueous acetone extracts instead of the spectra of the entire organism. It was felt that the use of several different extractions yielded more reliable results than the use of whole cells.

Kull and Grimm (1956 b) found that positive identification of bacteriophage can not be made by infrared analysis alone. Six serologically different "M" bacteriophages were distinguished by an exaggerated absorption at 1735 cm^{-1} which was not observed with the purified tobacco mosaic virus or E. coli "T" phages.

III. MATERIALS AND METHODS

Materials

Microorganisms

The following strains of Salmonella pullorum which were used in this study were obtained from the Communicable Disease Center, United States Public Health Service, Chamblee, Georgia: CDC 3522/51; NJ 1-40127; NJ 242-58; Mont 4232-57; WVa 533-58; Minn 607-58; and Ky 5159-57. In addition, an avirulent strain of S. pullorum was obtained from the Pullorum Disease Center, University of Maine, Orono. This culture was designated NEC 17.

The fermentative and biochemical reactions of these organisms were characteristic for S. pullorum as described by Breed et al. (1957). These organisms were agglutinated by antisera prepared for Group D of the Kaufman-White scheme as well as by antisera prepared against S. pullorum.

The cultures were streaked on Salmonella-Shigella agar (BBL) plates and isolated colonies were selected for transfer to trypticase soy agar slants to be maintained as stock cultures. The stock slants were stored at room temperature and weekly transfers were made.

Growth Medium

The medium used for the growth of organisms was that reported by Dooley (1955) for the production of large num-

bers of cells. The composition of the medium is shown in Table 1. This medium was prepared, the pH adjusted to 7.2 and placed in a 20 l carboy. The carboy containing the medium was autoclaved at 20 pounds pressure for 35 minutes.

Inocula

Inocula for the carboys were prepared from stock cultures by inoculating tubes of brain heart infusion broth (Difco). After 12 hours of incubation at 37 C, 1 ml of this culture was used to inoculate a 500 ml flask of trypticase soy broth (BBL). This flask was incubated at 37 C for 12 hours and used as the inoculum for the carboy.

Animal inocula of bacterial cells were prepared from 24 hour trypticase soy agar cultures. The organisms were washed from the agar and suspended in sterile water. The cell suspension was diluted to a reading of 120 to 130 on the Klett-Summerson photocolorimeter using the #66 filter. Dilutions were prepared from this standardized suspension.

Endotoxins were prepared for injection into chicks by weighing the necessary amount of endotoxin and adding sterile water. The suspension was mixed thoroughly until no particles were visible.

The chicks were injected on the right side of the abdomen immediately above the yolk sac. All challenging doses were administered in 1 ml amounts.

TABLE 1

COMPOSITION OF THE MEDIUM
USED FOR THE GROWTH OF
SALMONELLA PULLORUM

Constituent	Amount
Distilled water	10.0 l
Glucose (Tech)	200.0 g
Casamino acids (Tech)	200.0 g
Yeast extract	100.0 g
$K_2HPO_4 \cdot 3H_2O$	70.0 g
KH_2PO_4	30.0 g
NH_4Cl	50.0 g
$(NH_4)_2SO_4$	10.0 g
$MgSO_4$	1.0 g
$KHCO_3$	20.0 g
Sodium citrate	5.0 g
$CaCl_2$	0.2 g

Experimental Animals

One to three day old White Leghorn (Babcock strain) cockrels, hatched from eggs from pullorum-free flocks, were used in all experiments. Food and water were made available 12 hours after inoculation and *ad libitum* thereafter. The feed consisted of antibiotic-free breeder ration.

Methods

Extraction of Endotoxin

One of the earliest techniques for extraction of endotoxins was the trichloroacetic acid extraction of Boivin *et al.* (1933). It has been used extensively and is known to yield a highly toxic product with a high percentage of polysaccharide and a low percentage of nitrogen. It has been used for the extraction technique throughout this study.

After 48 hours growth at 37 C the cells were harvested by centrifugation in a Sharples centrifuge. The cells were washed once in distilled water and three times in cold acetone. The residual acetone was removed after the final washing by evaporation.

The acetone-killed and dried cells of *S. pullorum* were suspended in distilled water which had been cooled to 2 C. The concentration of cells was 1 g dry weight of cells to 25 ml of water. An equal volume of 0.5 N trichlo-

roacetic acid, also cooled to 2 C, was added to the cell suspension and the mixture was held at 2 C for two hours with frequent shaking.

The cells were removed by centrifugation, and the supernatant was dialyzed against running tap water for 24 hours to remove the acid. Ethyl alcohol was added to the dialyzed supernatant to a final concentration of 60 per cent alcohol. The precipitate was held at 2 C for 12 hours and subsequently collected by centrifuging at 1800 X G. After the precipitated endotoxin was washed once with cold ethanol, it was dried *in vacuo* over calcium chloride in a tared petri dish. After it was dried, the endotoxin was weighed and the infrared spectrum of each extraction made.

LD_{50} Determination of Viable Cells

The LD_{50} determinations of the various strains of *S. pullorum* were made according to the technique of Reed and Muench (1938). Five groups of eight chicks each were used in the determinations.

Cell suspensions were prepared in sterile water by the use of standard dilution procedures. The number of viable organisms was determined by plate count, in duplicate, by the use of the spread plate method. The plates were incubated at 37 C and counted after 36 hours.

Endotoxin Toxicity Studies

The toxicity of the endotoxins of strains NEC 17 and 3522/51 of S. pullorum was measured by determining the LD_{50} of each preparation. The endotoxins were dissolved in sterile water for administration. The determination was based on four or five groups of six chicks each. The inoculum was contained in 1 ml.

The toxicity of strains NJ 242, Mont 4232, WVa 533, Minn 607 and Ky 5159 was determined approximately by injecting each of six chicks with 5 mg of endotoxin.

Preparation of Whole Cells for Infrared Spectroscopy

The cells were grown in 50 ml quantities of trypticase soy broth (BBL, Lot # 9809) which had been filtered through a Seitz filter prior to sterilization. The cultures were incubated at 37 C for 24 hours. The cells were removed by centrifugation at 2000 X G and washed twice with distilled water. After washing, the cells were suspended in 0.5 to 1.0 ml distilled water and the suspension frozen in a thin layer along the wall of the tube by immersing the tube in a methanol-dry ice bath. They were held in the frozen state and lyophilized. After lyophilization the tubes were stored in a desiccator over calcium chloride until preparation for infrared spectroscopy.

Preparation of Tissue for Infrared Spectroscopy

One to two day old chicks were divided into four

groups randomly. The groups were normal control, fasted control, infected and intoxicated animals. The fasted controls received no food throughout the experiment, but received water 12 hours after the start of the experiment and ad libitum thereafter. The infected group received injections of approximately 10^4 organisms of S. pullorum 3522/51. The intoxicated birds received 5 mg of endotoxin prepared from S. pullorum 3522/51 60 hours after the injection of the infected group.

At 24, 48, 72 and 96 hours five birds from the infected and control groups were sacrificed and samples of the liver and the whole spleen of the birds removed. Approximately equal weights of the livers of the five birds were pooled, homogenized in a modified Potter-Elvehjem homogenizer and lyophilized. The spleens were treated similarly. Intoxicated birds were sacrificed at 72 hours after the injection of the infected birds. Tissue samples were pooled from groups of three birds in the case of the intoxicated group. The lyophilized tissue was stored in stoppered tubes over calcium chloride in a desiccator until preparation for infrared spectroscopy.

Infrared Spectroscopy

Solid phase sampling was utilized for the preparation of samples for infrared spectroscopy throughout this work. The potassium bromide pellet technique reported by

Schiedt and Reinwein (1952) and by Stimson and O'Donnell (1952) was used with minor modifications.

Spectrographic grade potassium bromide (Merck, Darmstadt, Lot # 53336N) was dried at 110 C and ground in a mullite mortar and pestle to a mesh of 300. After the potassium bromide was sieved it was placed in a weighing bottle and stored over calcium chloride in a desiccator. To prepare a pellet 199 mg of potassium bromide, prepared as described, was placed in a mullite mortar and pestle and mixed by grinding with 1 mg of the sample. The mixture was ground by hand for approximately three minutes. It was then transferred quantitatively to a pellet die (Hilger & Watts Ltd.), evacuated for five minutes and subjected to 20,000 pounds pressure under vacuum for five minutes in a Carver laboratory press. Following the same procedure reference pellets were prepared using 200 mg of potassium bromide with no sample.

Spectra were recorded using a Perkin-Elmer Model 137 Infracord spectrometer. Since this is a double beam instrument, a plain potassium bromide pellet was placed in the reference beam while the sample pellet was in the sample beam. The instrument was adjusted to 90 per cent transmittance at 4.5 microns, set for automatic slit width adjustment, and run at a speed of 1 micron per minute.

IV. RESULTS

The Endotoxin

Infrared spectra of the replicate extractions of the endotoxins of Salmonella pullorum strains 3522/51 and NEC 17 indicate that a satisfactorily uniform product was obtained by this extraction procedure. Figure 1 shows a typical spectrum of the preparations of each strain of organism. There were no major differences in the spectra.

The spectra of the endotoxins of the other strains of S. pullorum are shown in Figures 2 and 3.

Figure 4 is a detailed presentation of parts of the spectra of all the endotoxins which seem significant. The wavelengths presented are 4.0 to 4.5 microns, 5.6 to 5.9 microns, and 6.7 to 7.2 microns. These regions showed significant differences when compared on the basis of toxicity.

The average yield of endotoxin from strain 3522/51 was 207 mg per 5 g dry weight of cells, which represents 4.14 per cent of the mass of the cell. This average is based on three extractions. The average yield of four extractions of strain NEC 17 was 147 mg per 5 g dry weight of cells, or 2.94 per cent.

Viable cell LD_{50}'s

The LD_{50} of each strain of S. pullorum is shown in Table 2. The value for strain 3522/51 is based on four ex-

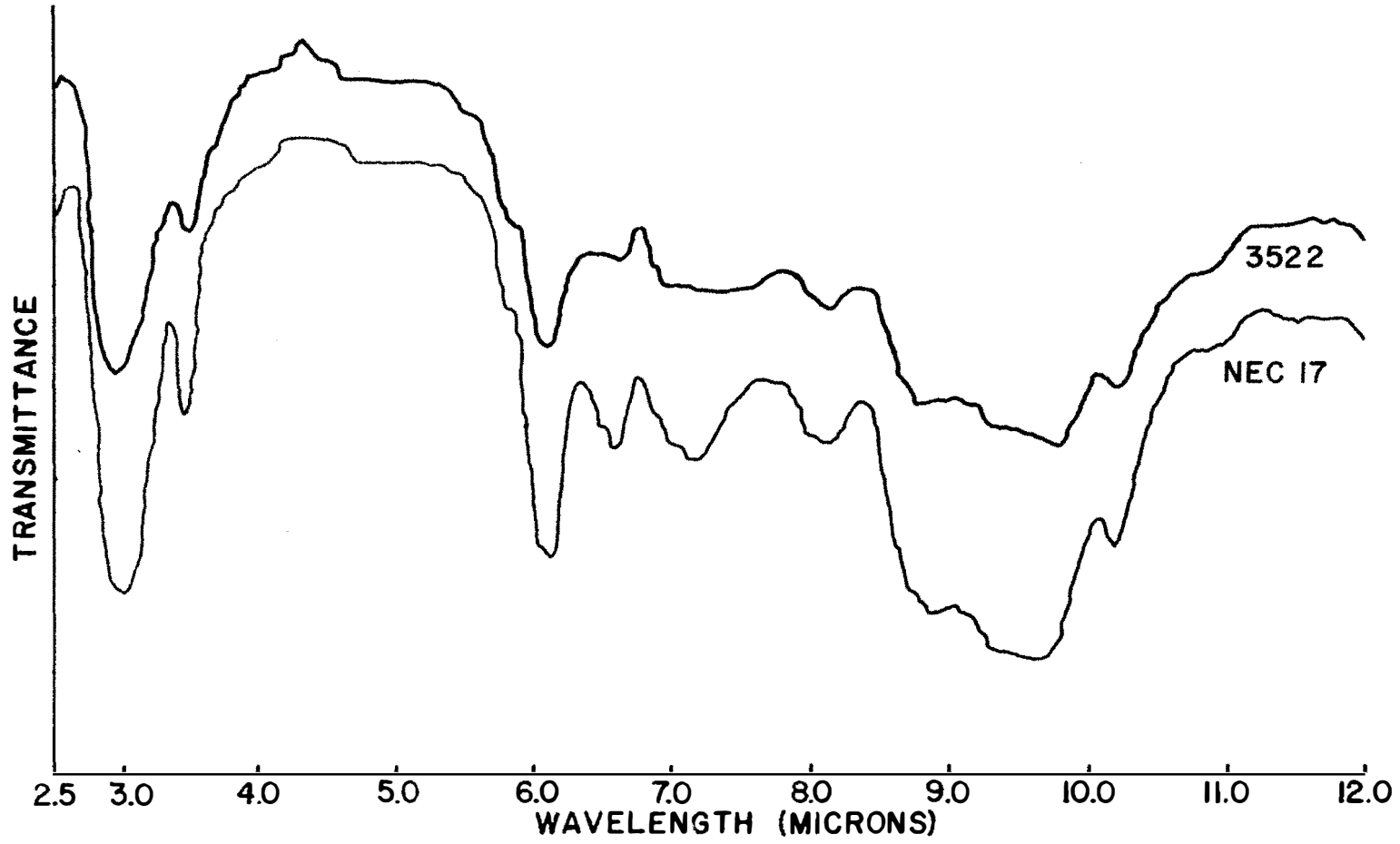

Figure 1. Infrared absorption spectra of the endotoxins of Salmonella pullorum, 3522/51 and NEC 17.

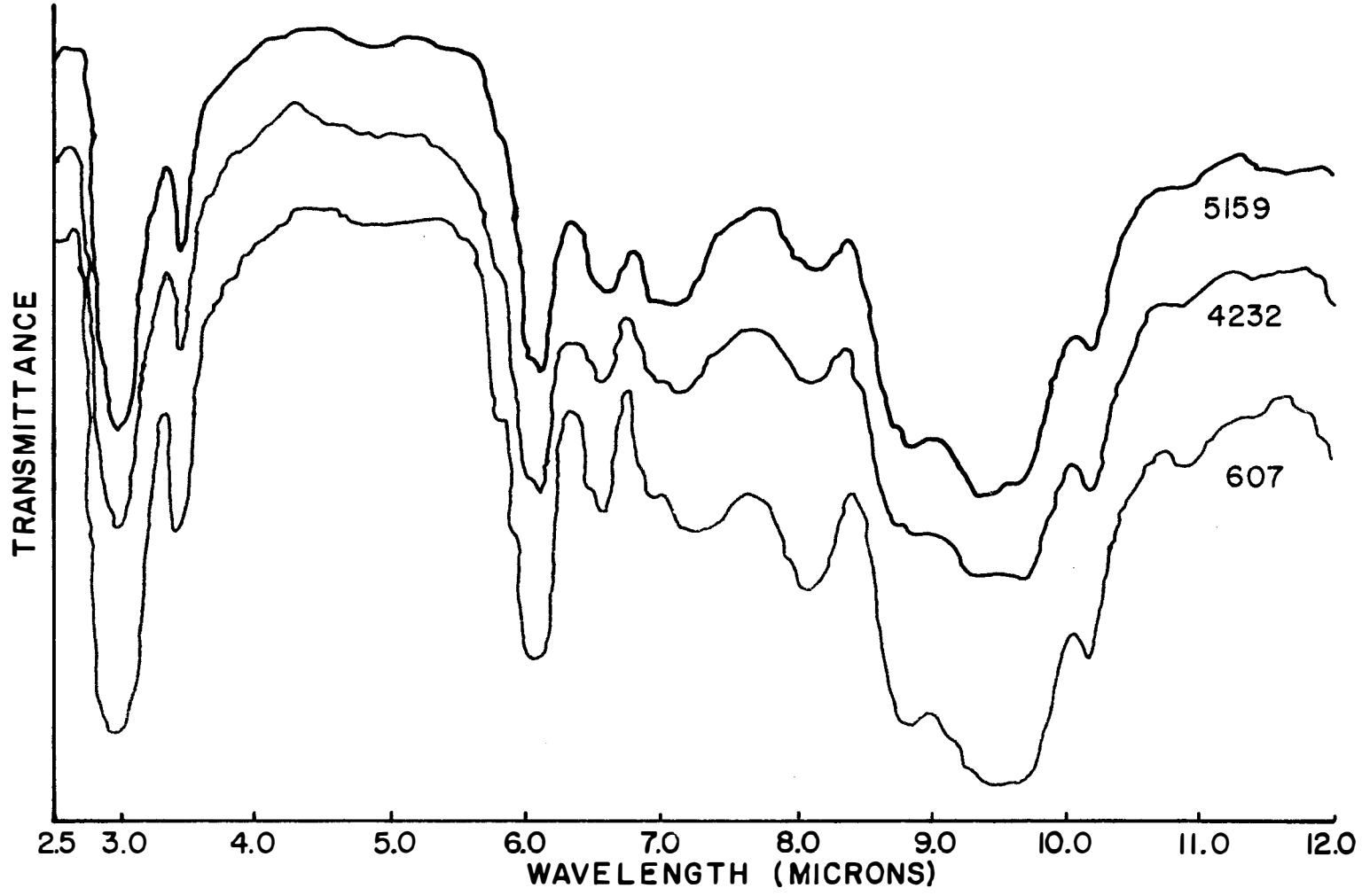

Figure 2. Infrared absorption spectra of the endotoxins of Salmonella pullorum, 5159, 4232 and 607.

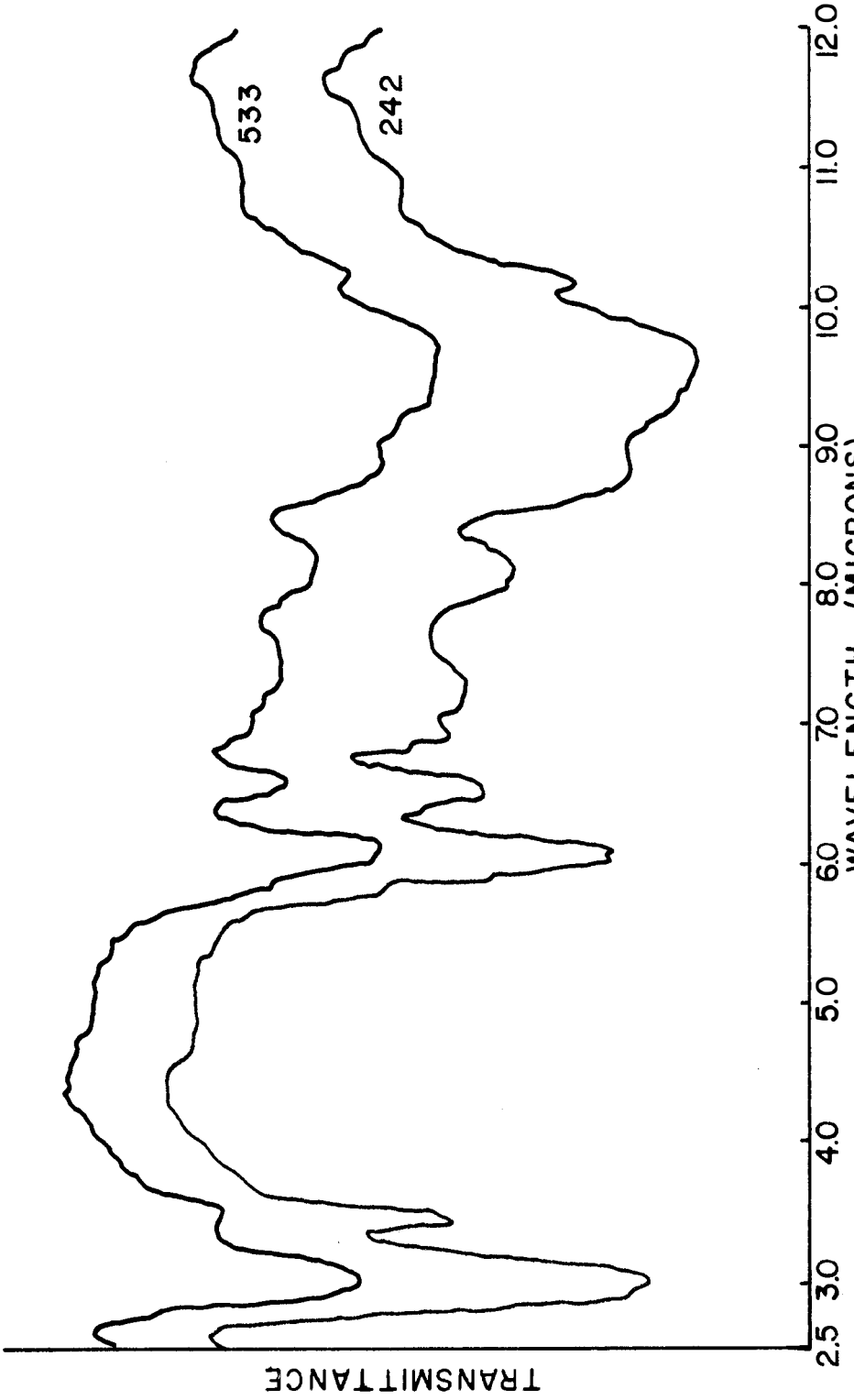

Figure 3. Infrared absorption spectra of the endotoxins of Salmonella pullorum, 242 and 533.

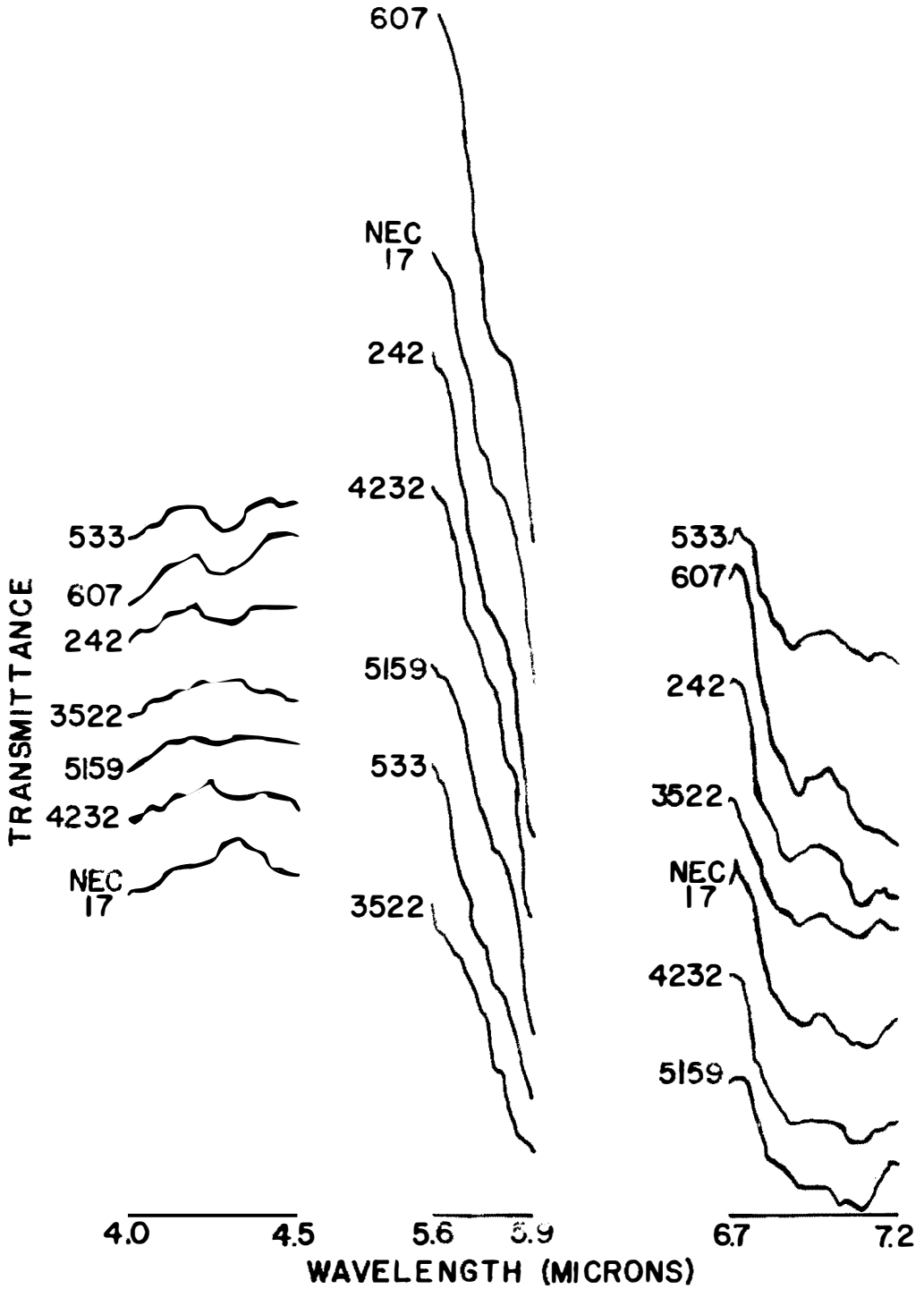

Figure 4. A detailed presentation of the significant portions of the infrared spectra of the endotoxins of seven strains of Salmonella pullorum.

TABLE 2

THE LD_{50} VALUES OF VARIOUS STRAINS OF SALMONELLA PULLORUM AND THE TOXICITIES OF THEIR ENDOTOXINS

Strain	Viable cell LD_{50}	Toxicity dead/survivors
3522/51	$10^{2.90}$	6/0
NEC 17	Avirulent	5/1
242	$10^{2.29}$	6/0
533	$10^{2.26}$	6/0
607	$10^{2.32}$	5/1
4232	$10^{3.28}$	1/5
5159	$10^{3.75}$	2/4

tractions and is similar to that reported by Ross et al. (1955) and by Dooley (1955). Strain NEC 17 failed to induce an infection when introduced into chicks in numbers as high as 10^8 and 10^9 organisms. The remainder of the values are the average of two determinations.

Toxicity Studies of the Endotoxin

The average LD_{50} of the endotoxin of strain 3522/51, based on two determinations was 3.5 mg per 35 g chick. The LD_{50} of strain NEC 17 was 4.5 mg per chick.

The approximate toxicities of the endotoxins of the other strains of S. pullorum are shown in Table 2. The various strains of organisms may be grouped by comparing the approximate toxicities. The endotoxins of strains 4232 and 5159 are significantly less toxic than are the preparations of the rest of the strains.

Infrared Spectroscopy of Whole Bacterial Cells

There were no significant differences between the spectra of the whole cells of the strains of S. pullorum used in this investigation. Some very minor differences were noted which were of no greater magnitude than those inherent in the technique and in the instrument. A typical spectrum of a whole cell preparation of S. pullorum is presented in Figure 5.

Infrared Spectroscopy of Chick Tissues

The spectra of liver tissues of normal, normal

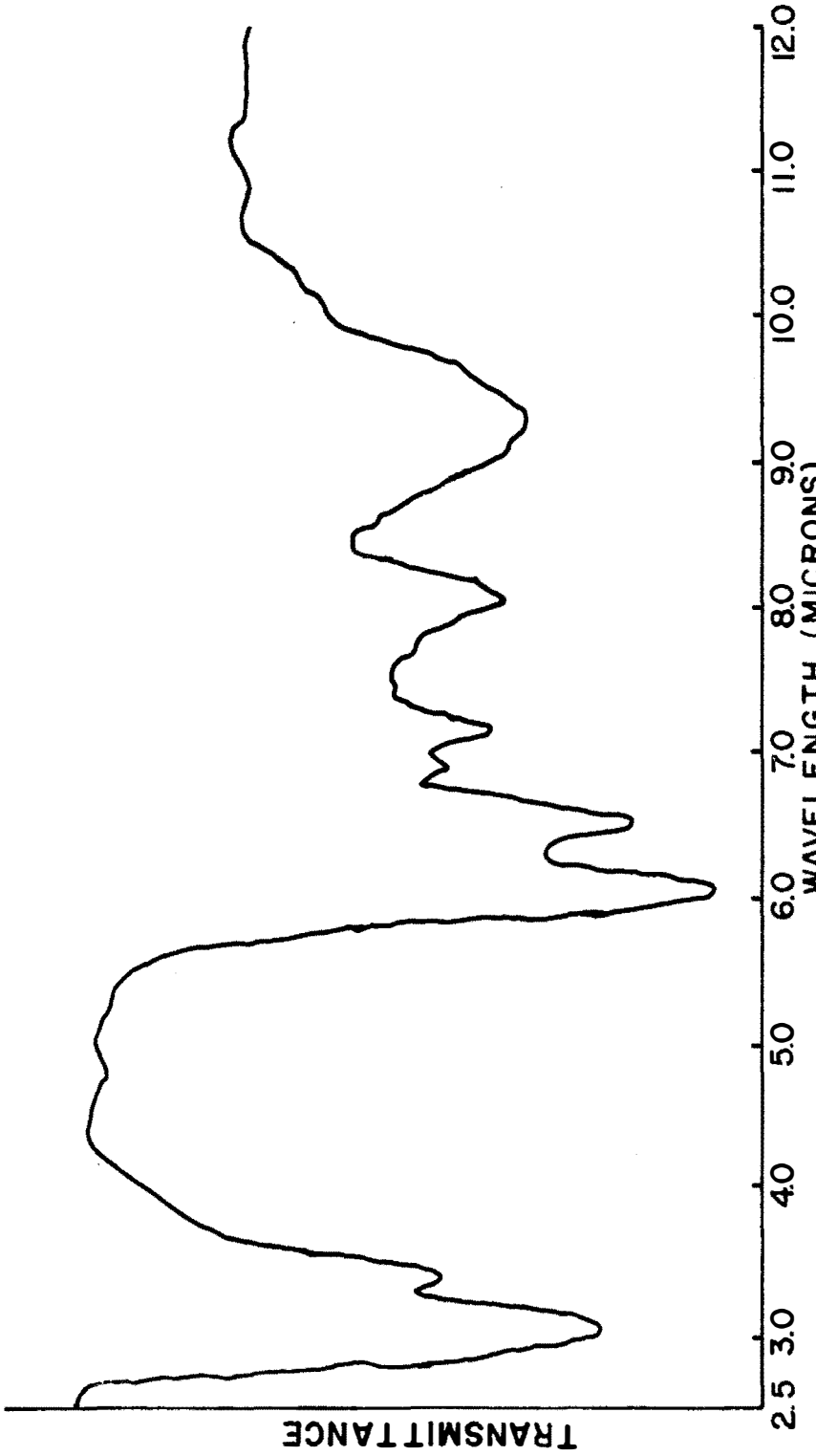

Figure 5. A typical infrared spectrum of whole cells of Salmonella pullorum.

fasted, infected and intoxicated chicks are shown in Figures 6 and 7. Figure 6 also presents the spectrum of the total lipid extracted from the liver of a normal chick. The tracing of the lipid spectrum was kindly furnished by Mr. Charles D. Jeffries. The endotoxin was administered 60 hours after infection; thus these spectra were made from tissues of a chick 12 hours after the introduction of endotoxin. This procedure enabled a comparison of tissues of chicks of the same age. Figure 8 shows, in detail, the major differences in these spectra at 72 hours after infection. Some differences in the spectra of tissues of normal chicks as they aged in the test interval were noted. The spectra of tissues from normal chicks at 12, 24, 72 and 96 hours after the beginning of the experiment are shown in Figure 9.

The spectra of the spleens of the normal, normal fasted, infected and intoxicated chicks are shown in Figure 10. The only difference noted is the presence of a small peak at 5.8 microns in the spectrum of the spleen of the intoxicated chick. No major spectral changes were noted in the spleen of normal chicks during the experimental time interval.

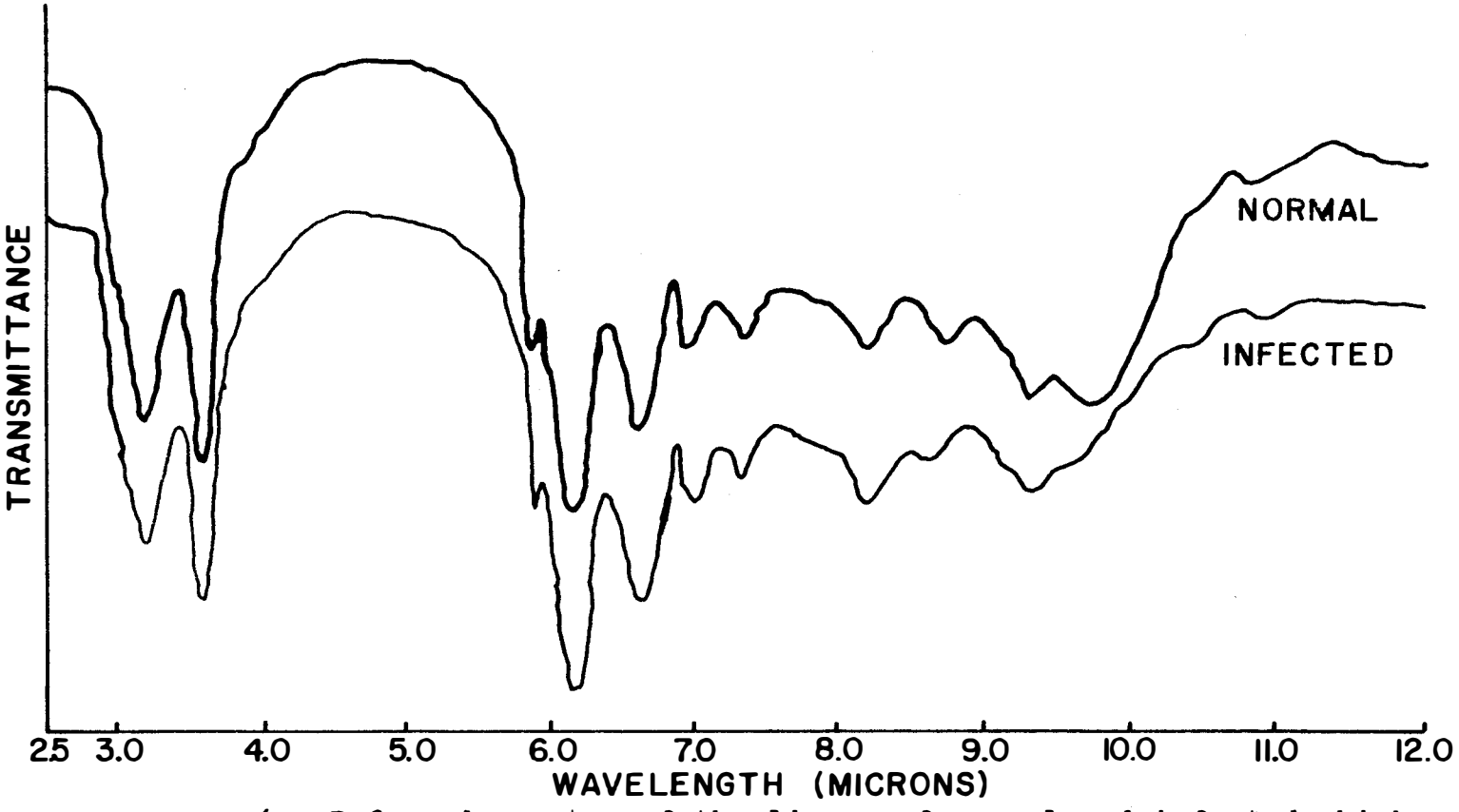

Figure 6. Infrared spectra of the livers of normal and infected chicks.

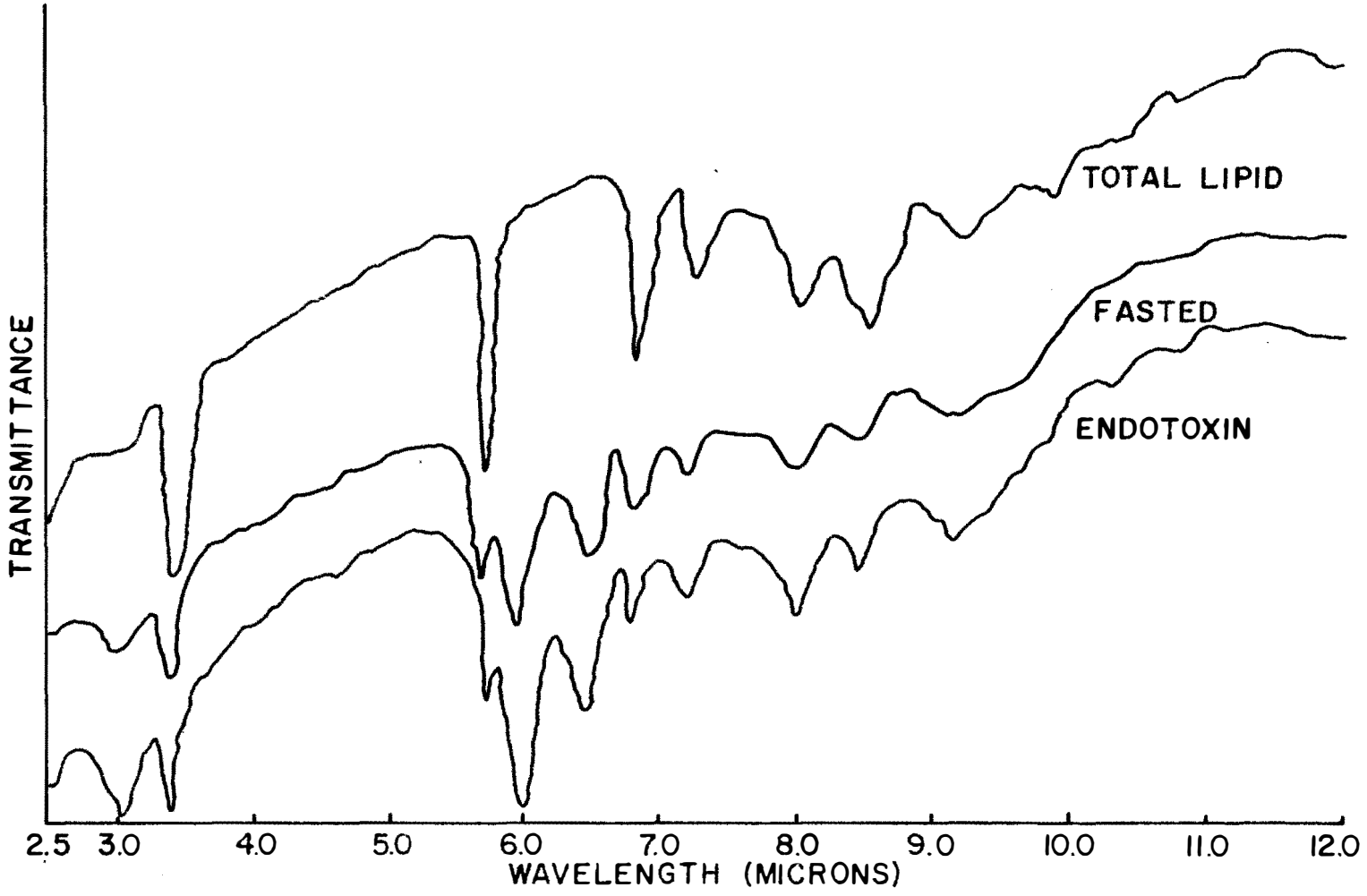

Figure 7. Infrared spectra of the livers of normal fasted and intoxicated chicks and of the total lipid of a chick liver.

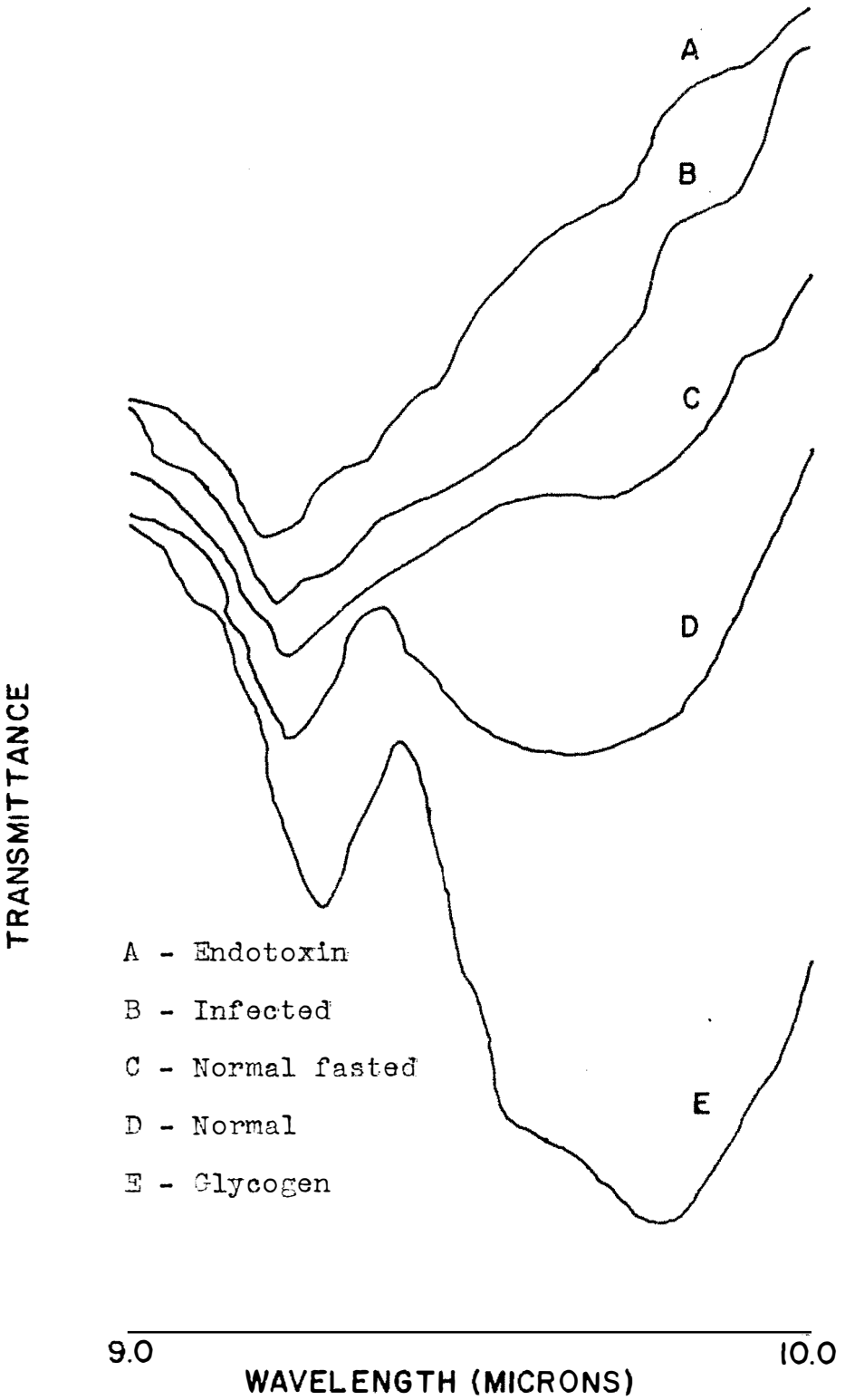

Figure 8. The infrared spectra of normal, normal fasted, infected and intoxicated chick livers and of glycogen.

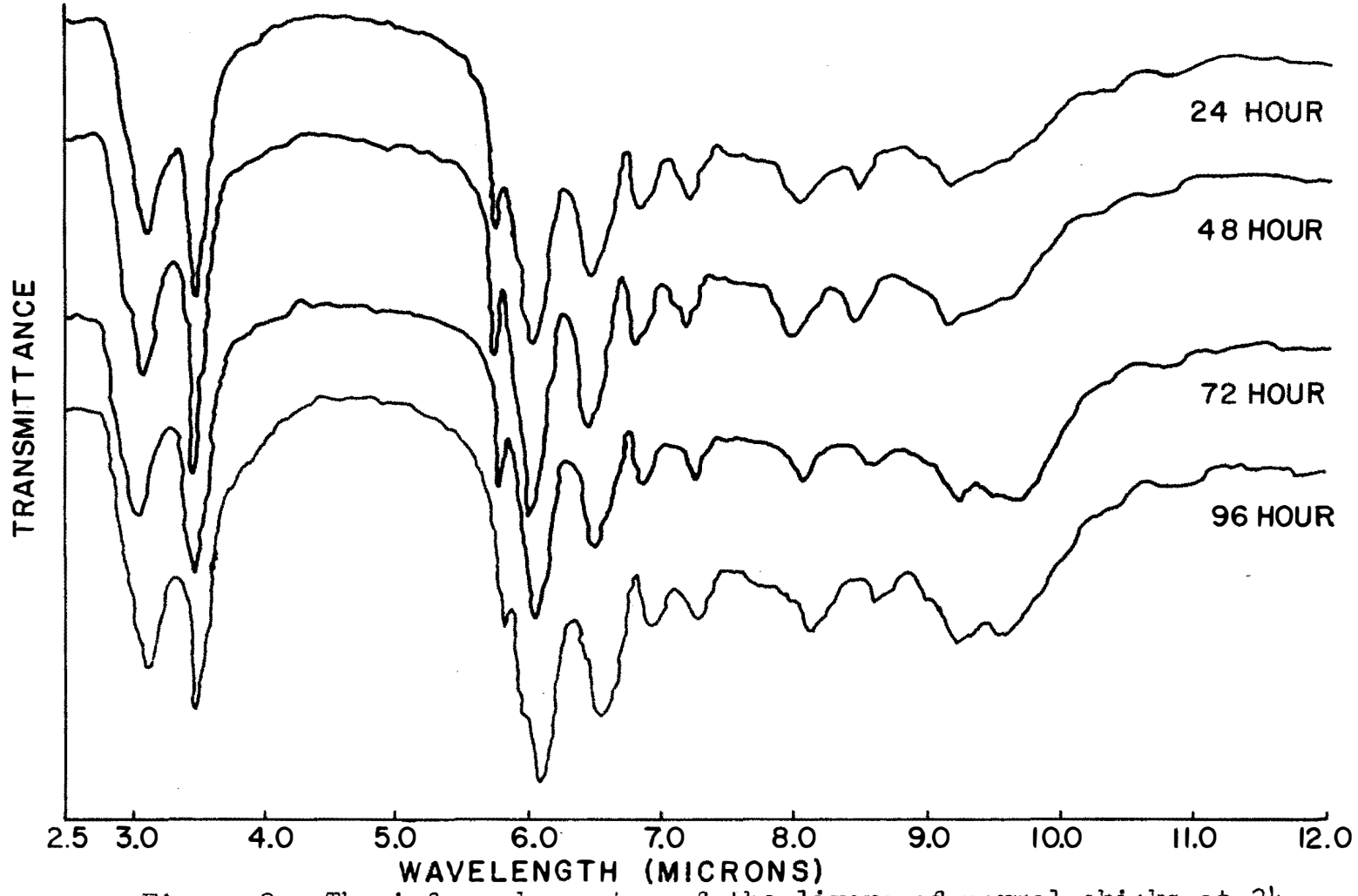

Figure 9. The infrared spectra of the livers of normal chicks at 24, 48, 72 and 96 hours.

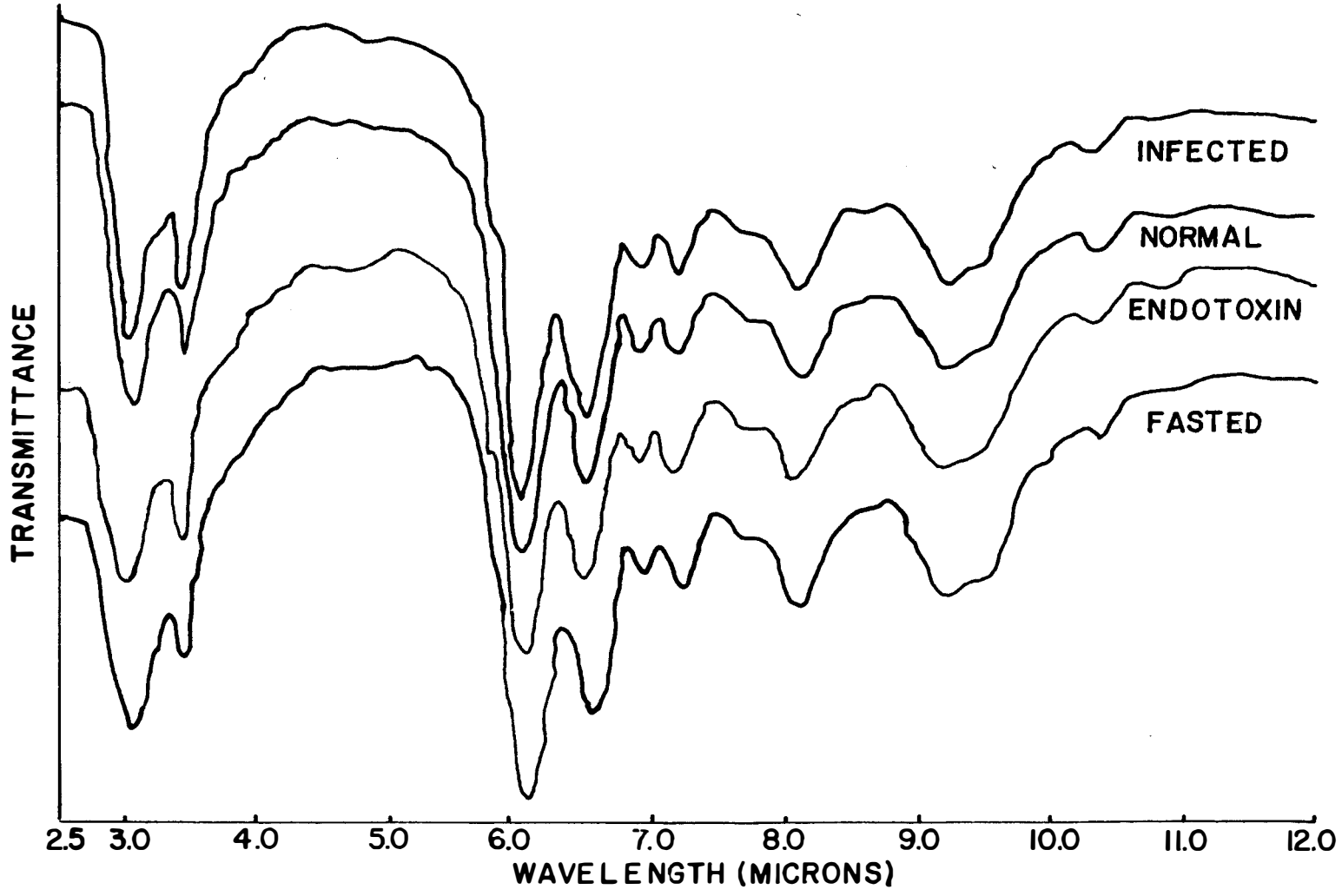

Figure 10. The infrared spectra of the spleens of normal, normal fasted, infected and intoxicated chicks.

V. DISCUSSION

Past investigations of endotoxins have relied primarily on the study of highly virulent strains of gram negative organisms. There have been only two reports of the investigation of both virulent and avirulent strains of the same species of microorganisms (Spink and Anderson, 1954; Boor and Miller, 1944). This study is concerned with the examination not only of highly virulent and avirulent strains of Salmonella pullorum, but with strains which have an intermediate virulence as well.

In this study the findings of Spink and Anderson (1954) and of Boor and Miller (1944) regarding the toxicity of endotoxin from virulent and avirulent strains of the same organism have been substantiated. The endotoxins of strains 3522/51 and NEC 17 of S. pullorum were of the same magnitude regarding toxicity while the viable cells of these strains differ widely in their virulence. This comparison of toxicity is based upon the administration of equal weights of the endotoxin. If the assumption is made that the other factors contributing to virulence of the organism are identical, then the difference between these strains could stem from a difference in the amount of endotoxin each cell is capable of producing. The findings of Shipitsina and Yemel'yanova (1956) in their investigation of the endotoxin of Pasteurella tularensis indi-

cate that this may, indeed, be the case. If, as in their findings, the avirulent cells contain one-half as much endotoxin as do the virulent cells, the body defenses of the host may well have an opportunity to combat the infection before a sufficient level of endotoxin was reached to cause death, since twice as many organisms would be required for this level to be attained. In this case the avirulent strain NEC 17 contains 71 per cent as much endotoxin as does the virulent strain 3522/51 per unit of dry weight. If the number of organisms of strain 3522/51 is arbitrarily assigned a value of 100, strain NEC 17 would have to attain approximately 41 per cent more organisms to produce this amount of endotoxin. Gilfillan (1956) found that the number of cells of S. pullorum 3522/51 increases to a maximum of about 10^9 in the carcass of the chick at about 48 hours. If this number is assumed to be lethal, strain NEC 17 would have to attain a population of 410,000,000 more organisms to cause death. While this many cells might be attained, it is conceivable that the host would be able to overcome the infection before that number was reached.

The observation that introducing a number of organisms of strain NEC 17 which is almost equivalent to the lethal number of strain 3522/51 fails to induce a lethal infection lends support to the concept that strain NEC 17 is not capable of multiplying to a lethal number in the

chick. The introduction of large numbers of cells of gram negative bacteria, either viable or killed, produces symptoms quite similar to those of purified endotoxins. Thus, if a sufficiently large number of S. pullorum strain NEC 17 cells were introduced into the chick, the effects could not be distinguished from those of the endotoxin.

It appears, therefore, that the difference in virulence of strain NEC 17 and strain 3522/51 is not due to a qualitative difference in the endotoxins of these two strains. The difference appears to be due to a quantitative difference in the endotoxins of the two strains and to the inability of strain NEC 17 to multiply effectively in the host.

As pointed out by Freter (1956), the assumption that there is only one toxic fraction associated with the bacterial cell may be faulty. The techniques used in this study, like those of other workers, are sufficiently harsh to result in the inactivation of many compounds and to denature proteinaceous materials. Among the cellular constituents it is possible that there are other highly toxic compounds. The absence of such a compound could also account for the lowered virulence of strain NEC 17.

A comparison of the virulence of the viable cells and the toxicity of the endotoxins of the other strains of S. pullorum does not confirm the findings with strain

NEC 17. A direct relationship appears to exist between the virulence of these other strains and the toxicity of their endotoxins. These strains are either highly virulent, as in the case of strains 242, 533 and 607, or of intermediate virulence, as in the case of strains 4232 and 5159. It is likely that these strains would be more similar to each other than they would to a completely avirulent strain, thus introducing fewer variables in comparing strains. The endotoxins of these organisms differ in degree of toxicity, rather than in being either toxic or non-toxic, as shown by the fact that deaths occurred among all groups. Thus it appears that the reason for the reduced virulence of strain NEC 17 differs from the cause of reduction of virulence in the other strains.

Since such a difference exists between strains of reduced virulence, the experimental approach of comparing a highly virulent strain to a completely avirulent one seems to have little significance in the investigation of the nature of pathogenic microorganisms. By comparing a number of highly virulent strains with strains of lesser virulence, but which are still virulent to some degree, a more accurate picture of the factors contributing to virulence would be obtained. The interpretation of such results would, of necessity, be more difficult since a quantitative approach rather than a qualitative one is neces-

sary.

The infrared spectroscopic examination of the endotoxins of these strains of S. pullorum was preceded by an investigation of the spectra of the whole cells. Reports in the literature indicated the possibility of differentiating between strains of this organism (Levine et al., 1953 b). If these differences could be correlated to the virulence of the organisms and the type of compound absorbing at that wavelength identified, the factors causing virulence might be approached more logically. This possibility was not fulfilled. From the results of this study the possibility of differentiating between strains of the same species of bacterium by infrared spectroscopy appears to be limited. A widely varying mutant might be differentiated by very careful techniques using a research grade instrument. The use of this technique for rapid differentiation is questionable. These findings do not preclude the possibility of using the technique on a species or genus level or of using extraction techniques in conjunction with infrared spectroscopy. The use of an instrument designed for control work is not suitable for such a study since it does not have sufficient resolution or flexibility.

The infrared spectroscopic comparison of the endotoxins from all the strains studied reveals rather uniform

preparations in their gross aspects. Some information regarding the nature of the preparation may be obtained by examining the absorption peaks. The absorption at 3.0 microns may be attributed to the presence of two molecular configurations. Amines are known to absorb strongly in this region, suggesting the presence of a protein fraction. Hydroxyl groups are also reported to absorb strongly in this area. The strong absorption seen in these spectra is probably due to the combined absorption of these two configurations. The band at 3.43 microns is indicative of the presence of methyl groups in the complex. The strong absorption at 6.0 to 6.1 microns is due to the presence of the carbonyl group in the peptide linkage of protein. The peak at 6.55 microns is attributed to the N-H deformation characteristic of the peptide linkage. When considered with the peaks at 3.0 and 6.0 to 6.1 microns, this peak substantiates the presence of protein in the endotoxin. The peaks occurring between 9.0 and 10.0 are considered generally characteristic of polysaccharides. The strong absorption in this region of these spectra confirms the presence of a large amount of polysaccharide in the complex.

Any difference in the infrared spectra will be of a minor nature since the comparison of these strains reveals varying biological properties of viable cell virulence and toxicity of the endotoxins. Such differences, while not

as striking as readily apparent ones, may be as significant as major differences. Spectral differences are considered significant if they are above the level of the instrument noise.

Figure 3 presents the differences which may be considered significant. These spectra were recorded on a Perkin-Elmer Model 21 double beam instrument which has appreciably better resolution than does the Model 137. In the spectra of the endotoxins of strains 242, 533 and 607 a small peak is readily apparent at 4.2 microns. This peak is absent, or greatly reduced, in the endotoxins of strains 3522/51, NEC 17, 4232 and 5159. Thus there appears to be little or no correlation of this absorption with the toxicity of the preparations. This absorption frequency has been tentatively assigned to the molecular configuration of phosphates.

The absorption at approximately 5.8 microns appears in the spectra recorded on the Model 137 spectrometer to correlate well with the biological properties of the preparations. This was not borne out in the spectra recorded on the Model 21 instrument. This point is very difficult to interpret in spectra of this nature, since there is a very strong absorption band at 6.1 microns which tends to mask lesser ones at nearby wavelengths. There is a definite indication of several peaks in the region of 5.8 microns.

An absorption band has been reported in the spectra of toxic preparations of endotoxins of various organisms (Webster et al., 1955; Dooley, 1955; Ormsbee and Larson, 1955). The absence of as strong an absorption in the spectra of these preparations as the other reported spectra indicates the possibility that the configuration responsible for this absorption is not essential in the quantities indicated by the spectra. The absorption frequency has been designated as characteristic of either the ester configuration or the carbonyl of the carboxyl group of the lipid moiety. The confusing nature of the fine structure of the spectrum in this region indicates the desirability of expanding the spectrum in this region to determine whether or not a correlation could be made with biological properties.

The spectra of the preparations of strains 3522/51, NEC 17, 242, 533 and 607 show an absorption at 6.9 microns which is greater than the absorption in the spectra of strains 4232 and 5159. This appears to be well correlated with the toxicities of the endotoxins. Absorption at this wavelength is difficult to assign to a specific molecular configuration. Colthup (1950) reports absorption by alkane groups and by hydroxyl groups at this wavelength. Absorption by alkanes at this wavelength is reported to be as intense and is accompanied by a weaker absorption at 7.2 to 7.4 microns. This does not fit the absorption shown in

these spectra as well as does the absorption by the hydroxyl group. Absorption peaks have been reported at 3.0, 6.9, and 9.2 to 10.0 microns to be characteristic of the hydroxyl group of primary alcohols. The peaks at 3.0 and 9.3 to 10.0 microns are found in the spectra of all the preparations at about the same intensity. These bands are not of value in these spectra since the absorption at 3.0 microns is due not only to this configuration, but also to the presence of the amine group. The broad absorption in the region from 9.0 to 10.0 microns is characteristic of polysaccharides and is so broad that it is of relatively little value for a specific identification.

The presence of a greater number of hydroxyl groups in the more toxic preparations may be due to a difference in the polysaccharide moiety of the complex. While the constituent sugars of the polysaccharide have been determined, the amounts and arrangement of the sugars in endotoxins have not been elaborated. A change in either of these factors could account for the presence of more free hydroxly groups and for the alteration in toxicity.

A considerable amount of research involving the infrared spectroscopy of animal tissues has been reported. This work has involved, primarily, the tissues of mammals. There have been no reports regarding the infrared spectra of avian tissue. The spectra of normal chick livers show

some changes as the chick ages. The principal change is the occurrence and increasing intensity of an absorption peak at 9.72 microns. This peak has been attributed to the presence of glycogen by Schwartz et al. (1954) who reported decreases in the glycogen levels of the livers of irradiated rats. The accumulation of glycogen in the liver of the normal chick is to be expected, as the metabolism of the chick is converted from the utilization of the yolk to the utilization of the ration supplied. In the infected chick the storage of glycogen does not occur. This may be attributed to either or both of two reasons: (1) the chick utilizes more energy than normal in resisting the disease, and (2) the endotoxin is known to affect glycogen levels. Figure 7 shows that the endotoxin is capable of interfering with the storage of glycogen. Kun (1948) has shown that the endotoxin of the meningococcus inhibits the synthesis of glycogen. Under these conditions the glycogen present is rapidly depleted. In the case of the intoxicated or infected chick the synthesis is inhibited before an appreciable amount has been accumulated. The normal fasted chick is deprived of an external source of protein and carbohydrate and is thus required to consume most of the available carbohydrate as it is formed from the contents of the yolk. A very minor amount of glycogen is apparent from the spectrum of the fasted liver.

The effects of the infection upon the synthesis of glycogen can not be attributed primarily to a cessation of feeding by the infected birds. While the intake of food is reduced drastically, it is not stopped completely until the animal is prostrate, at which time the effects of the endotoxin of the organism are greater, and more rapid, than is the effect of fasting.

A comparison of the spectra of the livers of the intoxicated and the normal fasted animals with that of the total lipids from the liver of a chick shows a striking similarity in the decreased transmittance at lower wavelengths. Thus it appears that the level of fat in the liver has increased measurably in these groups. An examination of the absorption peaks shows that all the peaks, however, are present in these that occur in the normal or infected animals. The occurrence of a fatty liver in a normal fasted chick probably is due to the fact that the chick has been on a high fat-low protein diet followed by starvation. Popper and Schaffner (1957) state that either of these nutritional imbalances may be responsible for fatty livers.

The occurrence of fatty infiltration of the liver of chicks injected with the endotoxin of S. pullorum was reported by Dooley (1957) who attributed it to the combined actions of several factors. These included: (1) increased

mobilization of depot fats; (2) a reduction in the transport of fats from the liver, and (3) impaired utilization of fats in the liver due to the reduction in the regeneration of adenosine triphosphate.

No changes were noted in the spleens of normal chicks during the experimental time interval. Only one minor difference was noted among the four experimental groups. This difference was the occurrence of a small absorption peak at 5.8 microns in the spectrum of the intoxicated chick spleen. The fact that absorption at this wavelength has been assigned as characteristic of esters indicates that a small amount of fat has accumulated in the spleen. These fats probably would occur as fatty acid esters in triglycerides.

VI. SUMMARY

The virulence of strains 3522/51, NEC 17, NJ 242, Mont 4232, WVa 533, Minn 607 and Ky 5159 of Salmonella pullorum was determined and compared with the toxicities of the purified endotoxins of these strains. The avirulent strain NEC 17 produced an endotoxin which had a potency of the same magnitude as that of the virulent strain CDC 3522/51. Strain NEC 17 produced a significantly less amount of endotoxin on a dry cell weight basis.

A comparison of a number of strains of S. pullorum of intermediate virulence indicated that the differences in virulence were due to differences in the endotoxin rather than to a reduced amount of endotoxin.

Infrared spectra of the endotoxins revealed only minor differences. The biological differences of the preparations were correlated with the presence of the absorption peak at 6.9 microns. The peak was tentatively identified as being characteristic of the presence of hydroxyl groups.

The infrared spectra of homogenized livers and spleens of normal, normal fasted, infected and intoxicated chicks were compared. Spectroscopic evidence showed that the glycogen level of the infected and intoxicated chicks was reduced drastically, while that of the normal fasted chick was reduced to almost the same level.

The occurrence of fatty infiltration of the liver

was noted in the case of the intoxicated and normal fasted chicks. No major changes in the infrared spectra of the spleens of the chicks were found.

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