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To the Graduate Council:

I am submitting herewith a thesis written by Elmo Sharber Dooley entitled "A Study of Some of the Metabolic Aspects of Host-Parasite Interaction Using the Baby Chick and Salmonella pullorum." I recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Bacteriology.

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33-A

A STUDY OF SOME OF THE METABOLIC ASPECTS OF HOST-PARASITE INTERACTION USING THE BABY CHICK AND SALMONELLA PULLORUM

A THESIS

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

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by

Elmo Sharber Dooley

June 1957

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E. S. D.

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TABLE OF CONTENTS

CHAPTER		PAGE
I.	INTRODUCTION	1
II.	LITERATURE SURVEY	4
III.	MATERIALS AND METHODS	12
	Materials	12
	Microorganism	12
	Reagents and chemicals	13
	Growth medium	13
	Inocula	15
	Dried cell preparations	15
	Endotoxin	16
	Experimental animals	16
	Methods	17
	Extraction of endotoxin	17
	Chromatographic procedures	18
	LD_{50} determinations	19
	Experimental period	19
	Determination of amino acids in the blood and liver	20
	Treatment of the chicks with amino acids	21
	Determination of body temperatures	22
	Blood chemistry	22
	Preparation of the protein-free blood filtrates	22

CHAPTER PAGE

III. (continued)

Total non-protein nitrogen (NPN) 22

Blood uric acid 22

Blood creatinine 23

Blood ammonia 23

Blood sugar 23

Hemoglobin 23

Phosphorus 23

Determination of citrate in the liver 23

Coenzyme A determinations 23

Treatment of chicks with cortisone 24

Statistics 24

IV. RESULTS 25

Growth of the microorganism 25

The endotoxin 25

Chick LD_{50} determinations 30

Symptoms and pathology of intoxication by endotoxin 30

Amino acid profiles in the blood and liver 33

Profiles of arginine in intoxicated and infected chicks 39

Profiles of glycine in intoxicated and infected chicks 40

Profiles of methionine in intoxicated and infected chicks 41

CHAPTER PAGE

IV. (continued)

Profiles of tryptophan in intoxicated and infected chicks 42

Effect of endotoxin on the body temperature of chicks 43

Changes in the blood chemistry of chicks during intoxication and infection 43

Concentrations of citrate in the livers of intoxicated and infected chicks 51

Coenzyme A levels in the livers of intoxicated and infected chicks 51

V. DISCUSSION 56

VI. SUMMARY 78

BIBLIOGRAPHY 82

LIST OF TABLES

TABLE		PAGE
1.	Composition of the Medium Used for the Growth of *Salmonella pullorum*	14
2.	Some Qualitative and Biochemical Characteristics of the Endotoxin of *Salmonella pullorum*	26
3.	Amino Acids Present in the Protein Fraction of the Endotoxin of *Salmonella pullorum* as Determined Chromatographically	27
4.	Chick LD_{50} of *Salmonella pullorum*	31
5.	Chick LD_{50} of the Endotoxin of *Salmonella pullorum* . .	32
6.	Effect of the Administration of Amino Acids on the Survival Time of Chicks Intoxicated with the Endotoxin of *Salmonella pullorum*	34
7.	Effect of Cortisone on the Body Temperature (F.) of Chicks Inoculated with 0.5 mg of the Endotoxin of *Salmonella pullorum*	45
8.	Non-protein Nitrogen, Urea and Creatine in the Blood of Chicks Intoxicated with the Endotoxin of *Salmonella pullorum* and Infected with *Salmonella pullorum* . . .	46
9.	Uric Acid, Ammonia, and Hemoglobin Concentration in the Blood of Chicks Intoxicated with the Endotoxin of *Salmonella pullorum* and Infected with *Salmonella pullorum*	47

TABLE PAGE

10. Blood Sugar and Inorganic Phosphorus Concentrations of Chicks Intoxicated with the Endotoxin of *Salmonella pullorum* and Infected with *Salmonella pullorum* . . . 48

11. Coenzyme A and Citrate Levels in the Livers of Chicks Intoxicated with *S. pullorum* or Infected with *S. pullorum* Taken at Twenty-four Hours . . . . . .

12. Blood Ammonia Levels in Chicks Intoxicated with the Endotoxin of *S. pullorum* or Infected with *S. pullorum* at Thirty-six Hours 53

13. The Effect of Cortisone on the Survival Time of Chicks Intoxicated with the Endotoxin of *S. pullorum* or Infected with *S. pullorum* 54

14. Effect of the Administration of Arginine on the Blood Ammonia and Urea Levels of Chicks Intoxicated with the Endotoxin of *S. pullorum* and Infected with *S. pullorum* at Twenty-four Hours 55

LIST OF FIGURES

FIGURE PAGE

1. Infrared absorption spectrum of the endotoxin of *Salmonella pullorum* obtained by using the Perkin-Elmer Model 12C spectrometer 28
2. Infrared absorption spectrum of the endotoxin of *Salmonella pullorum* obtained by using the Perkin-Elmer Model 21 spectrometer 29
3. Typical chromatograms of the free amino acids in the blood of normal and intoxicated chicks 35
4. Typical chromatograms of the bound amino acids of the blood of normal and intoxicated chicks 36
5. Typical chromatograms of the total amino acids (free and bound) of the livers of normal and intoxicated chicks 37
6. Body temperature of chicks inoculated with 0.5 mg of *Salmonella pullorum* endotoxin 44
7. Effect of the administration of endotoxin on the hemoglobin concentrations of chicks intoxicated with *Salmonella pullorum* endotoxin 50
8. A proposed hepatic scheme relating amino acid metabolism and nitrogen excretion in chicks intoxicated with *Salmonella pullorum* endotoxin 65

CHAPTER I

INTRODUCTION

Many symptoms of infectious disease are now generally recognized as resulting largely from an interaction of chemical substances from the host and parasite. This concept has followed the establishment of the germ theory of disease. Experimentally, the concept has also provided a useful approach to the study of the biochemical basis of the host-parasite relationship.

The concept of chemical substances as the determinants of disease implies the production by the parasite of diffusable compounds toxic to the host. Roux and Yersin (1888) first noted that bacteria could produce toxins capable of duplicating many of the symptoms of disease. For convenience, these workers classified these compounds as endotoxins and exotoxins according to whether they were found inside or outside the parent organism. Pasteur, in his classical studies with fowl cholera, came close to the biochemical basis of infection. He observed that the toxic manifestations of this disease could be produced in normal animals by the injection of cultural filtrates of the causative agent, Pasteurella multocida.

The attempt to relate physiological alterations of infection with metabolic disturbances within the host represents the new approach to the problem of describing the biochemical basis of infection. Dubos (1955a, b) is one of the current leaders who has rekindled interest in the "biochemical determinants" of infection. He has described this as

one of the most pressing problems facing investigators of disease at the present time. Evidence in support of this approach must first come from detailed studies of the host-parasite relationship in the natural environment.

Pullorum disease of chicks has been shown to be well suited for the investigation of an infection in its natural environment. The chick is the natural host of Salmonella pullorum and its tissues are well adapted for the growth of this microbe. Results obtained from a study of this natural relationship could present an accurate picture of the metabolic alterations accompanying the infectious process.

The observations of Rettger (1909), indicating the presence of unabsorbed yolk in young chicks dying of pullorum disease, first suggested an impairment of metabolic processes. Recent studies in this laboratory have provided an explanation for some of the physiological changes produced by this disease. It has been shown that the nitrogen excretion pattern, amino acid metabolism, and the intermediary carbohydrate metabolism of the chick are altered during experimental infection with S. pullorum (Ross et al., 1955a, b, 1956; Gilfillan et al., 1956a, b).

Following the isolation of an endotoxin lethal for chicks from S. pullorum (Dooley and Holtman, 1956), it became of interest to determine the role of this preparation in the pathology of pullorum disease. This dissertation will describe the influence of endotoxin upon certain phases of nitrogen excretion, amino acid metabolism and selected reactants of the intermediary carbohydrate metabolism of chicks. Profiles of various essential amino acids, nitrogen excretion

products, enzymes and the intermediate, citrate, have been studied. An attempt has been made to relate the results of this study to those previously obtained in this laboratory and to a general understanding of the infectious process.

CHAPTER II

LITERATURE SURVEY

A survey of the scientific literature reveals very few papers concerned with the mode of action of endotoxins in altering the metabolic processes of man or animals. There are, however, numerous observations of physiological changes induced by the injection of gram-negative bacterial vaccines and endotoxins.

The manifestations of toxicity by endotoxins and bacterial vaccines have been shown to include the following: (a) hyperthermia followed by hypothermia, (b) extreme polymorphonuclear leucopenia followed by leucocytosis, (c) the production of hemorrhagic necrosis in the substance of rapidly growing tumors, (d) with small doses, the rapid disappearance of a state of resistance against the same and other endotoxins, (e) profound vasomotor disturbances which may terminate in shock characterized by general arteriolar constriction, and (f) disturbances in metabolism consisting of hyperglycemia, followed, in some cases, by hypogylcemia, abrupt depletion of liver glycogen, and excessive amounts of lactic and citric acids in the blood and tissues. Inorganic phosphorus in the blood also increases. Endotoxins are Shwartzman active substances when injected in properly spaced doses. Burrows (1951) and Thomas (1954) have reviewed the various physiological and pharmocological effects produced by endotoxins.

Conspicuous manifestations of disturbances in the carbohydrate metabolism have been reported by many investigators. The most

frequently reported effect of intoxication by bacterial toxins has been that of hyperglycemia followed by extreme hypoglycemia in the terminal phases. Menten and Manning (1924) reported hyperglycemia in rabbits and guinea pigs during spontaneous salmonella infections. Subsequently, Zwecker and Goodell (1925) made similar observations in animals receiving injections of heat-killed gram-negative bacteria. The observation of hyperglycemia followed, in some cases, by hypoglycemia has been widely observed and reported (Menten and Manning, 1925; Menten, 1926; Evans and Zwecker, 1927; Delafield, 1932, 1934; Boivin and Mesrobeanu, 1934; Wein, 1938; Cameron et al., 1940; Oddy and Evans, 1940; DeRenzi and Grasselini, 1940; Koch and Olitzki, 1946; Penner and Klein, 1952; Takeda et al., 1955; and Weil and Spink, 1956).

Other observations indicating disturbances in the carbohydrate metabolism during intoxication with endotoxin have included alterations in the glycogen reserves, lactic and pyruvic acid levels in the blood, and in the inorganic phosphorus levels of the blood. Glycogen reserves have been shown to be depleted by the time the hypoglycemia is established (Cameron et al., 1940; Lithander, 1945; Olitzki et al., Fox et al., 1947; Kun, 1948; Kun and Abood, 1949; and Boquet et al., 1956).

Lactic acid content of the blood and tissues has been found to increase following the injection of gram-negative vaccines and endotoxins (Boivin and Mesrobeanu, 1936; Kun and Miller, 1948; Thomas and Stetson, 1949; and Takeda et al., 1955). This increase in the lactic

content of the blood and tissue was studied in detail by Kun and Miller (1948). In the terminal stages of intoxication, the content of pyruvate became markedly reduced. It was observed that succinic dehydrogenase activity was inhibited in the muscle and liver of intoxicated animals. Cytochrome oxidase activity was not effected by the endotoxins of the meningococci and Salmonella typhimurium. It was suggested, on the basis of these observations, that the effects of endotoxin might be accounted for in terms of induced tissue anoxia. An increased concentration of lactate has also been observed in skin areas injected twenty-four hours previously with the endotoxin of the meningococci or Serratia marcescens (Thomas and Stetson, 1949).

Kun (1948) found that the formation of glycogen from pyruvate and glucose was completely inhibited in vivo by rat liver slices in the presence of the endotoxins of the meningococci and S. typhimurium. The amount of glycogen formed in vitro by liver slices was also reduced by endotoxin. The inhibition could be reversed by the addition of zinc-free insulin. These findings seemed to indicate that the activity of hexokinase was inhibited by endotoxin.

The literature contains only a few papers concerned with the effects of endotoxins upon the amino acid metabolism and nitrogen excretion patterns of man or experimental animals. Reports of physiological alterations in patients undergoing non-specific fever therapy involving the use of gram-negative vaccines, however, are numerous in medical literature. Hench (1932) discussed the physiological reactions following such therapy. Changes were noted in the

basal metabolism and blood pressure. Alterations in the peripheral and splanchnic and vasomotor mechanisms were frequently seen. Changes in the size and permeability of the arteries and capillaries were marked. Concentrations of urinary nitrogen, phosphorus, urea, and uric acid increased. The sedimentation rate and the total non-protein nitrogen of the whole blood and serum increased. Sugar tolerance and the blood albumin/globulin ratio was altered. The activity of the liver, spleen, and gastrointestinal tract varied from normal. Hektoen (1935) also noted an increase in the non-protein nitrogen of patients receiving injections of a gram-negative bacterial pyrogen.

Holmes (1939) in a review of the subject of toxemia, suggested that since the three and four carbon compounds arising from the utilization of amino acids are indistinguishable from the fractions arising from the metabolism of carbohydrates and lipids, it would be illogical to expect the effects of toxemia to be confined to any specific group of metabolites. In spite of this suggestion, however, very little attention was given to the study of amino acid or lipid metabolism during intoxication.

The observations of Favorite and Morgan (1942) indicated that the total protein nitrogen, urea nitrogen, creatine, chloride, glucose, and the carbon dioxide combining power of the blood of animals receiving *S. typhosa* endotoxin were unchanged. Pathological changes were noted, however, in the heart muscle, bone marrow, and vascular beds.

The plasma protein shifts observed by earlier workers were confirmed by Kopp (1942). He found that patients undergoing

non-specific fever therapy, involving the use of typhoid vaccine, demonstrated shifts in the globulin and decreases in the serum albumin. Fibrinogen content of the blood also varied during the treatment.

The body temperature and non-protein nitrogen of dogs and guinea pigs were found to be increased by the injection of the polysaccharide of S. marcescens (Franke, 1944). The blood pressure decreased during intoxication and adrenalin was ineffective for the restoration or maintenance of normal levels. Necroscopy revealed extensive intestinal congestion and hemorrhage.

Bonetti (1945) found that the excretion of total nitrogen and histidine was accelerated during the early stages of fever induced by the injection of a bacterial pyrogen. The excretion fell below normal levels as the body temperature returned to normal. Normal excretion patterns were reached at approximately four hours following the end of the fever.

A slight increase in the blood uric acid levels has been observed in rabbits with Brown-Pearce carcinoma and treated with S. marcescens polysaccharide. There was no significant increase in the blood proteose and glomerual filtration decreased in the experimental animals (Westfall and Dunn, 1946).

In studies with hyman psychotic patients, Atschule et al., (1950) found that the injection of typhoid vaccine produced changes in the urinary output of 17-ketosteroids. The urinary uric acid/creatine ratio was found to vary inversely with the size of the dose of the vaccine.

These isolated reports from the literature serve to emphasize the need for a further study of the mechanisms whereby endotoxins alter the amino acid metabolism and nitrogen excretion patterns in man and animals. The apparent metabolic changes might be the direct result of the vascular reaction to endotoxin which leads to a state of tissue anoxia. There can be little doubt, however, that alterations in the nitrogen metabolism of the host contribute to the general state of intoxication and that such alterations are important in the final outcome of the host-parasite relationship.

In a series of studies designed to determine the role of tricarboxylic acid cycle in infection Berry and Mitchell (1953a, b, 1954c) and Berry et al. (1954a, b) observed an accumulation of citrate in the tissues of mice infected with S. typhimurium and treated with fluoroacetate. Similar increases in the injection of suspensions of heat-killed organisms and by the injection of other inhibitors of the tricarboxylic acid cycle including malonate.

Gilfillan et al. (1956a, b) found that the injection of malonate fluoroacetate, citrate, arsenite and succinate reduced the survival time of chicks infected with S. pullorum. Citrate was found to accumulate in the livers of both the infected and normal chicks treated with fluoroacetate. The results were comparable in magnitude of increase and induction time with those obtained in rats treated with the same inhibitor (Lindenbaum et al., 1951).

The reports of increased concentrations of citrate in the tissues of animals infected with gram-negative micro-organisms and

intoxicated with endotoxin revealed a biochemical disturbance not previously reported. The results, however, might have been anticipated in view of the earlier results obtained by Kun and Miller (1948) showing the inhibition of succinic dehydrogenase activity by endotoxins. Since infection, tricarboxylic acid cycle inhibitors, and endotoxins apparently inhibit the same enzyme system(s), these experimental results offered an explanation for the accumulation of citrate in the organs of experimentally intoxicated or infected chicks.

Ross et al. (1955a, b, 1956) found that the nitrogen excretion pattern and amino acid metabolism of the chick were altered by infection with S. pullorum. The concentrations of arginine, glycine, methionine and tryptophan in the blood and liver were shown to be markedly reduced by the infection. Blood urea, creatinine and uric acid of the blood increased during the experimental infection. A seven-to-ten-fold increase in blood urea made this compound the principal nitrogen excretion product of the infected chick. Since it is known that urea in sufficient concentration is toxic for uricotelic animals, it appeared that the production of this substance was involved in the toxicity associated with pullorum disease. The possible involvement of endotoxin in the accumulation of the products of nitrogen excretion in the blood was suggested by these workers (Ross et al., 1956).

The investigations of Berry and his associates (1953a, b, 1954a, b, c), Gilfillan et al. (1956a, b) and of Ross and his associates (1955 a, b, 1956), as well as those of earlier workers, have shown that gram-negative infections are capable of altering the

carbohydrate and nitrogen metabolism of experimental animals. Endotoxins have been directly implicated in the impairment of carbohydrate metabolism. Although there is no direct evidence implicating endotoxins in the disturbances of nitrogen metabolism, a study of this aspect of intoxication presented a promising beginning for a valuable approach to the biochemical basis of infection.

CHAPTER III

MATERIALS AND METHODS

Materials

Microorganism

A highly virulent strain of Salmonella pullorum (CDC 3522/51; N. J. 1-40127; XII_2) obtained from the Communicable Disease Center, United States Public Health Service, Chamblee, Georgia, was used as the infectious agent and as the source of endotoxin throughout this study. The high state of virulence was maintained by successive transfer through chicks prior to use. Qualitative isolations of the organism were usually obtained from blood, heart, spleen, bone marrow, and liver. Small amounts of these tissues were streaked on Salmonella-Shigella agar (BBL) plates and these were incubated at 37 C for twenty-four hours. Typical colonies were picked from the plates and transfers were made to nutrient agar slants which following incubation were stored at 4 C. These slants served as stock cultures. Frequent isolations of the organism were made from experimental animals during the course of the investigation for identification according to this procedure.

The fermentative reactions of the organism were the formation of acid from rhamnose and the formation of acid and gas from xylose, dextrose, and mannitol. No change was observed in broth tubes containing maltose, lactose, dulcitol and salicin as the chief carbon sources. No indication of citrate utilization was obtained on agar cultures.

Hydrogen sulfide was formed.

The organisms were agglutinated by antisera prepared for Group D of the Kaufman-White scheme.

Reagents and Chemicals

Chemicals defined as "chemically pure" were used throughout this study except as indicated.

The amino acids utilized in animal inoculations and as chromatographic standards were obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio. The hydrochloride forms were used in solutions prepared on a molar weight basis in sterile glass distilled water.

A solution of heparin sodium, prepared by Eli Lilly and Company, Indianapolis, Indiana, was used as an anticoagulant. This preparation contained approximately 10 mg of heparin sodium per ml and 0.2 ml of the solution was added to the beakers used to collect blood.

A saline suspension of cortisone acetate, prepared by Merck and Company, Rahway, New Jersey, containing 25 mg of cortisone acetate per ml was used during the investigation.

Growth Medium

A semi-synthetic medium, similar to that described by Gilfillan et al. (1955), was used to grow the large quantities of cells for the extraction of the endotoxin. The composition of this medium is given in Table 1. The solutions were prepared and autoclaved separately with the exception of solution D which was sterilized by filtration.

TABLE 1

COMPOSITION OF THE MEDIUM USED FOR THE GROWTH OF *SALMONELLA PULLORUM*

Solution A		Solution D	
Dist. Water	7.5 l	Dist. Water	0.5 l
K_2HPO_4	180.0 g	$KHCO_3$	30.0 g
KH_2PO_4	45.0 g		
Solution B		**Solution E**	
Dist. Water	4.0 l	Dist. Water	1.0 l
Glucose (tech. grade)	300.0 g	NH_4Cl	75.0 g
		$(NH_4)_2SO_4$	15.0 g
		Sodium citrate	7.5 g
		$MgSO_4$	1.5 g
		$CaCl_2$	0.5 g
Solution C		**Solution F**	
Dist. Water	1.0 l	Dist. Water	0.5 l
Casamino acids (tech. grade)	300.0 g	Yeast extract	150.0 g

Solutions A, B, C, E, and F sterilized by autoclaving. Solution D sterilized by filtration. Combined volume 14.5 liters. Final pH adjusted to 7.3.

Prior to inoculation, the six solutions were combined aseptically in a sterile 20 l carboy and the final pH was adjusted to 7.3

Inocula

Inocula for the carboys were prepared from stock cultures by streaking to S-S agar (BBL) plates and incubating for twenty-four hours at 37 C. A typical colony was selected from the plates and used to inoculate a 500 ml flask of nutrient broth. The flask was incubated at 37 C for twenty-four hours. Prior to the addition of the contents to the carboy, aliquots were removed and used to determine visible counts of the inocula. From duplicate streak plates, the average number of viable organisms in the inocula was determined to be 5.8×10^7 per ml.

Animal inocula were prepared from twenty-four hour broth cultures of S. pullorum. Organisms were harvested by centrifugation, washed in saline, and resuspended in saline. Turbidity of the suspension was adjusted to a reading of 100 on the Klett-Summerson photocolorimeter and a 1:100 dilution of the adjusted suspension was prepared. Animals were injected intraperitoneally with one ml of the diluted suspension. Duplicate plate counts prepared from such a dilution indicated that the inocula contained approximately 10^3 viable organisms per ml.

Dried Cell Preparations

Wet cells from the carboys, harvested in the Sharples supercentrifuge, were collected and suspended overnight in acetone. On the

following morning the cells were collected by filtration and washed with fresh acetone. Care was taken to break up all clumps of cells. The killed cells were then placed in shallow dishes and dried *in vacuo* over calcium chloride. Following the drying for forty-eight hours, the cells were weighed and stored in petri dishes until used for the extraction of endotoxin.

Endotoxin

The specific gram-negative endotoxin of *S. pullorum* was prepared by a modification of the Boivin *et al.* (1953) technique. A description of the extraction technique will be presented under Methods. The physiochemical characterization of the endotoxin molecule will be presented in Chapter IV.

Experimental Animals

One to three day old White Leghorn cockerels, hatched from eggs from "pullorum-free" flocks, were used in all animal experiments. Prior to use the chicks were selected, pooled, and placed in groups at random. The average weight of the chicks was 35 g. Food and water were made available *ad libitum* during the experimental period. An antibiotic-free ration was provided which contained 18 per cent soya bean meal, 3 per cent corn gluten meal, 2 per cent fish meal, 4 per cent meat scraps, 23 per cent oats, 20 per cent barley, and 30 per cent wheat. This ration contained more than the amount of amino acids essential for the normal growth of the chicks.

Methods

Extraction of Endotoxin

A modification of the Boivin *et al.* (1933) extraction technique was selected because previous studies indicated that it yielded an endotoxin with a high percentage of polysaccharide and a low percentage of nitrogenous material. Acetone-killed and dried cells were suspended in distilled water at a rate of 25 ml per gram of dry cells and the resulting suspension was cooled to 2 C. An equal volume of a 10 per cent solution of trichloroacetic acid (TCA), previously cooled to 2 C, was added to the mixture and the flask was stored at 2 C for three hours with frequent shaking.

The cells were removed by centrifugation and the resulting opalescent solution was dialyzed against running tap water for twenty-four hours to remove the TCA. The toxic fraction remaining in the acid-free supernatant was precipitated by the addition of ethanol until a final concentration of 68 per cent ethanol was reached. The precipitate was collected by centrifugation at 1800 G and was washed three times with ethanol and dried *in vacuo*. The endotoxin, a white amorphous substance, was stored in the dry state until used.

A series of qualitative and biochemical tests were employed to characterize the endotoxin molecule. The total nitrogen was determined by micro-Kjeldahl digestion and nesslerization of the digest. Total carbohydrate was estimated as reducing power of glucose units. Total protein was calculated using the nitrogen factor of 6.25. Lipid was determined gravimetrically after hydrolysis and extraction with ether

and chloroform. Phosphorus was determined according to the method of Fiske and SubbaRow (1925). The ninhydrin test was used to detect amino acids following acid hydrolysis and chromatographic separation. The Biuret test for the peptide linkage and Millon's test for monohydroxy benezene derivatives were conducted on solutions of the endotoxin or on hydrolysates. A qualitative determination of the sugars composing the polysaccharide fraction of the endotoxin was accomplished by paper strip chromatography and comparison with standard sugar solutions.

The ultra-violet absorption spectrum of the endotoxin was obtained with the Beckman DU spectrophotometer. The per cent transmission was measured over a range of 200 to 330 millimicrons.

The infrared absorption spectra of the endotoxin were obtained by using both the Perkin-Elmer Model 12 C and Model 21 recording spectrometers. Mounts for the Model 12 C were prepared by air-drying an aqueous solution of the endotoxin on the surface of a sodium chloride window. A calcium fluoride prism was used over the range of 2 to 4 microns and a sodium chloride prism was used over the range of 5.5 to 13.5 microns in the Model 12 C spectrometer.

Mounts for the Model 21 recording spectrometer were prepared by grinding and mixing 1, 3, and 5 mg of the endotoxin with 400 mg amounts of optical grade potassium bromide (KBr) and pressing a pellet under a total pressure of 25,000 pounds. Scanning was carried out over a range of 2 to 15 microns using a sodium chloride prism.

Chromatographic Procedures

Free and bound amino acids of the blood serum and the amino

acids composing the protein molecule were determined by the two-dimensional paper partition chromatographic technique of Williams and Kirby (1948) by using the ascending capillary technique of Horne and Pollard (1948). Butanol-glacial acetic acid-water in a ratio of 4:1:1 and 83 per cent phenol in water were used as solvent systems. Ninhydrin was used as a color reagent and the amino acids were identified by comparing Rf values with those reported by Berry et al. (1951) and with the Rf values obtained by using solutions of known amino acids. Sheets were placed in the phenol-water solvent first, followed by the butanol-glacial acetic acid-water solvent.

An 83 per cent phenol in water mixture was used as the solvent in the determination of the sugars composing the poly-saccharide fraction of the endotoxin. Strips were sprayed with orcinol and with acid phthalate color reagents to identify the aldose and ketose sugars.

LD_{50} Determinations

The LD_{50} determinations for the endotoxin and for the experimental strain of *S. pullorum* were made according to the technique of Reed and Muench (1938). Suspensions of the endotoxin and the microorganisms were prepared in sterile physiological saline and were inoculated by the intraperitoneal route. Five groups of twelve animals each were used in the determinations.

Experimental Period

The mortality of chicks from pullorum disease is greatest during the first two weeks following hatching. Resistance of chicks increases

rapidly with age (Rettger et al., 1912). Infection with serious consequence seldom occurs after the third or fourth day of hatching. The most critical period of infection is the first forty-eight hours after hatching.

In newly hatched chicks the body temperature is approximately 100 F, but it has been shown that it rises during the first four days (Lamoreux and Hutt, 1939). At four days the mean body temperature of chicks kept under normal brooder conditions is more than 105.5 F in both the heavy and light breeds. After four days the body temperature of the light breeds has been shown to increase at a more rapid rate than that of the heavy breeds.

The experimental period for this investigation was confined to the three day period following hatching in order to study the chick during the period of greatest susceptibility and to minimize the effects of increased resistance associated with the genetically controlled factor of body temperature.

Determination of Amino Acids in the Blood and Liver

The free and bound amino acids were determined in blood samples from the experimental groups by the following procedure. The animals were decapitated and the blood was collected in small beakers. Following the collection of the blood, livers were excised, blotted dry, and quick frozen for future study. The sera were deproteinized with an aliquot of acetone and were then filtered. The filtrate was washed with additional acetone and the washings were combined with the original filtrate. For chromatographic analysis of the free amino

acids 0.2 ml of the filtrate was applied to the sheets.

The precipitate from the step above was used for the determination of the bound amino acids of the blood. An equal volume of concentrated hydrochloric acid was added to the precipitate after it was washed twice with acetone. The mixture was autoclaved at 15 pounds pressure and 120 C for two hours. The resulting liquid was evaporated to dryness and the original volume was restored by the addition of glass distilled water. The solution was neutralized by the addition of silver oxide. Following filtration, 0.2 ml of the filtrate was applied to the chromatographic sheets.

The total amino acids (free and bound) of the liver were determined by taking one gram of minced tissue and adding 2 ml of concentrated hydrochloric acid. After a thorough mixing the material was autoclaved at 15 pounds pressure and 120 C for two hours. The hydrolysate was evaporated to dryness, resuspended in 2 ml of glass distilled water, and neutralized with silver oxide. Following filtration, 0.2 ml of the filtrate was applied to the chromatographic sheets for analysis.

Treatment of the Chicks With Amino Acids

Amino acids employed in the tests were of the L-form. Both arginine and glycine were prepared by suspending 100 mg of the amino acid per ml of distilled water. Administration was by the intraperitoneal route. Injections of glycine were given at the start of the experiment and at intervals of twelve, twenty-four, thirty-six, and forty-eight hours following intoxication or infection.

Injections of arginine were made at the time of inoculation

with endotoxin or microorganisms and at intervals of twelve, twenty-four, and thirty-six hours.

Determination of Body Temperatures

The body temperatures of the experimental chicks were taken with a rectal mercury thermometer after insertion through the cloaca for one minute, following the technique of Lamoreux and Hutt (1939). Uniform depth of insertion was insured by a stopper placed on the thermometer at a point five-eighths of an inch from the tip of the bulb. To further insure uniformity, all temperatures were taken between 12:30 and 4:00 p. m. each day, which according to Lamoreux and Hutt (1939) is the period of peak diurnal temperature. Chicks were brooded at a temperature of 40 C during this investigation.

Blood Chemistry

Blood samples were collected in small beakers containing 0.2 ml of heparin sodium solution to control coagulation. The determinations were made according to widely accepted laboratory procedures.

Preparation of the protein-free blood filtrates. Hayden's (1923) modification of the Folin and Wu (1919) precipitation technique was employed in the preparation of protein-free blood filtrates.

Total non-protein nitrogen (NPN). The non-protein nitrogen of the blood was determined colorimetrically by the method of Koch and McMeekin (1924).

Blood uric acid. The blood uric acid was determined by the method of Brown (1926).

Blood creatinine. The blood creatinine was determined colorimetrically by the technique of Folin and Wu (1919).

Blood ammonia. The blood ammonia was determined by the microdiffusion technique described by Seligson and Seligson (1951).

Blood sugar. The blood sugar was determined according to Folin's (1929) modification of the Folin and Wu (1919) technique.

Hemoglobin. The hemoglobin was determined colorimetrically by the procedure described by Schultze and Elvehjem (1934). This technique was designed for use with avian blood and eliminates the difficulty of turbidity created by nucleated erythrocytes. Standard solutions of acid hematin, prepared by the technique of Elvehjem (1931), were used in the preparation of a standard curve.

Phosphorus. The serum inorganic phosphorus levels were determined by the method of Fiske and SubbaRow (1925).

Determination of Citrate in the Liver

The levels of citrate in the livers were determined by the pentabromacetone method as modified by Ettinger *et al.* (1952). A Beckman DU spectrophotometer was used for all readings. Tissue hemogenates were prepared according to the technique described by Gilfillan *et al.* (1956b).

Coenzyme A Determinations

The levels of coenzyme A in the livers were determined by multiplying the mg of pantothenate by 1.43 which represents the amount of pantothenate in one unit of coenzyme A (Olson and Kaplan, 1948).

Pantothenate was determined by microbiological assay using the technique of Skeggs and Wright (1944).

Treatment of Chicks With Cortisone

In the experiments involving the use of cortisone, the hormone was administered intraperitoneally in doses of 5.0 mg. Animals were given an injection twelve hours prior to the start of the experiment, at the time of challenging, and twelve hours following challenging.

Statistics

Group means were compared statistically where indicated. The technique of White (1952) was used as a test of significance in comparing the results obtained from the experimental groups. In these studies "P" was considered significant when the value was 0.05 or less.

CHAPTER IV

RESULTS

Growth of the Microorganism

The average yield from the carboys was 1.22 g dry weight cells per liter of medium. This yield was obtained by aeration and maintenance of the pH of the growth medium at 7.3 by periodic adjustments during the first twelve hours of growth. A total of 140 g of dried cells were grown and 2.5 g of endotoxin prepared for this investigation.

The Endotoxin

The results of the qualitative and biochemical tests conducted on the endotoxin or its hydrolysates are presented in Table 2.

The polysaccharide fraction of the molecule was found to be composed of mannose, galactose, rhamnose and hexosamine by chromatographic analysis.

Amino acids composing the protein fraction of the endotoxin molecule, as determined chromatographically, are presented in Table 3.

The ultra-violet absorption spectrum of the endotoxin showed no peaks characteristic of nucleic acids and was typical of the spectra obtained from turbid suspensions.

The infrared absorption spectra of the endotoxin are presented in Figures 1 and 2. In general, these spectra resemble those obtained from endotoxins of other gram-negative bacteria. The presence of protein, lipic and carbohydrate fractions in the molecule were confirmed by the spectra.

TABLE 2

SOME QUALITATIVE AND BIOCHEMICAL CHARACTERISTICS
OF THE ENDOTOXIN OF *SALMONELLA PULLORUM*

Carbohydrate (as glucose units)	50-60 per cent
Molisch test	Positive
Benedicts test	Positive
Selivanoff test	Negative
Protein (N x 6.25)	20-23 per cent
Nitrogen	3.2-3.6 per cent
Biuret test	Positive
Lipid (ether-chloroform extractable)	20-25 per cent
Phosphorus	1.8-2.3 per cent

TABLE 3

AMINO ACIDS PRESENT IN THE PROTEIN FRACTION OF THE ENDOTOXIN OF *SALMONELLA PULLORUM* AS DETERMINED CHROMATOGRAPHICALLY

Amino Acids	
Glutamic acid	Methionine
Aspartic acid	Arginine
Threonine	Phenylalanine
Serine	Leucine/ Isoleucine
Glycine	
Tyrosine	Valine
Alanine	Lysine

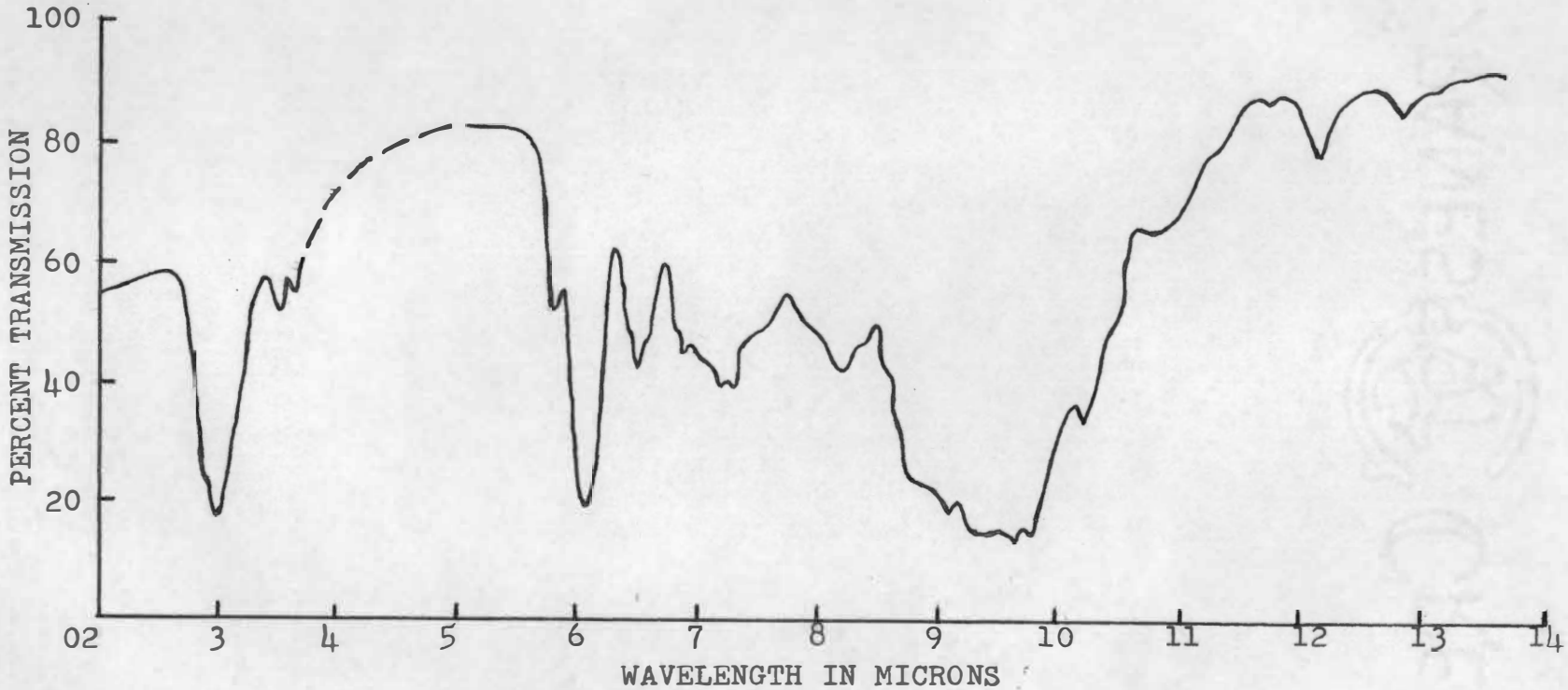

Figure 1. Infrared absorption spectrum of the endotoxin of Salmonella pullorum obtained by using the Perkin-Elmer Model 12C spectrometer.

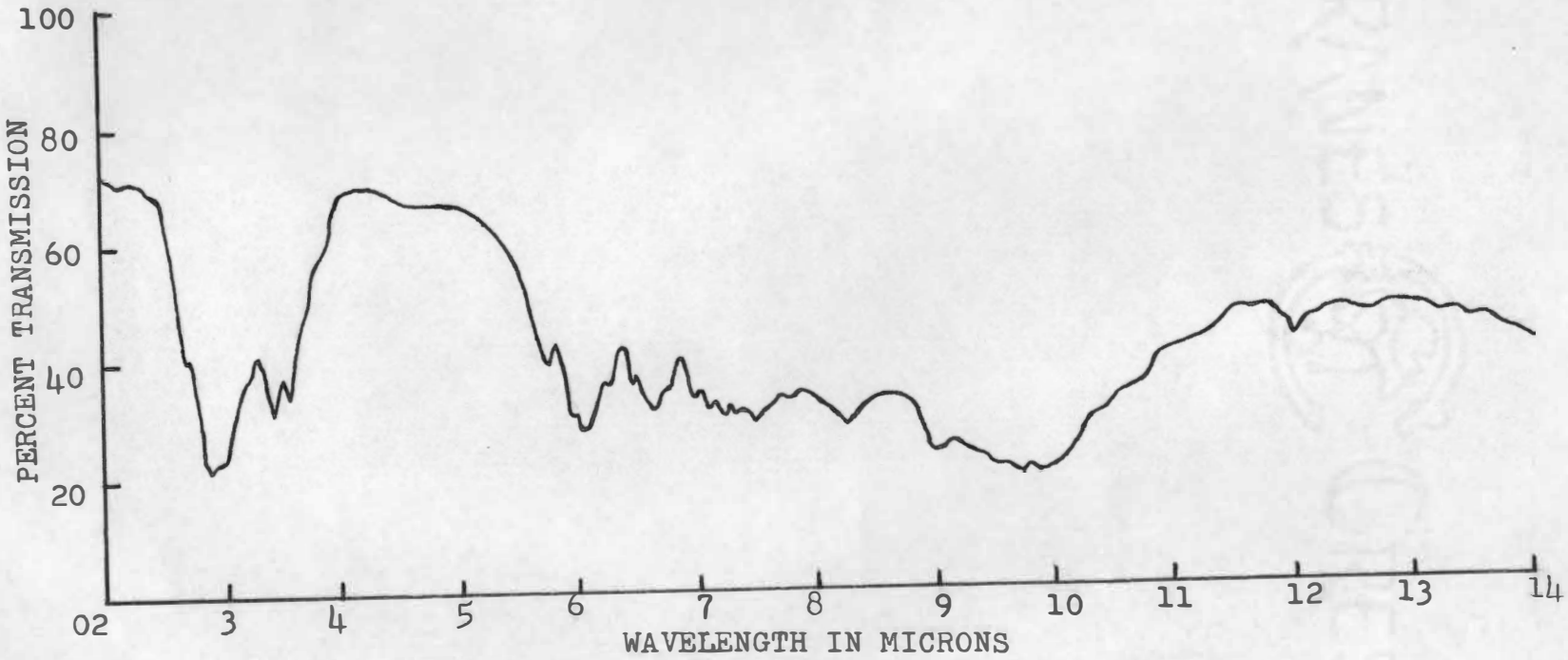

Figure 2. Infrared absorption spectrum of the endotoxin of Salmonella pullorum obtained by using the Perkin-Elmer Model 21 spectrometer.

Chick LD_{50} Determinations

The LD_{50} for the virulent strain of S. pullorum was calculated to be $10^{2.4}$ viable organisms when injected by the intraperitoneal route. This value was similar to those reported by other investogators for the experimental strain of S. pullorum (Ross et al., 1955a; Gilfillan et al., 1956a). The injection of 10^3 viable cells proved to be uniformly lethal for 35 g chicks (Table 4).

The LD_{50} for the endotoxin was calculated to be 6.4 mg (Table 5). Injections were made by the intraperitoneal route. The injection of 1.0 mg of the endotoxin was lethal for 20 g white mice.

Symptoms and Pathology of Intoxication by Endotoxin

The general toxic properties of the endotoxin derived from S. pullorum possessed, to a high degree, the toxic attributes of colonially smooth gram-negative bacilli. When administered intraperitoneally to the chicks the endotoxin was capable of producing fever, diarrhea, prostration, and death. The grossly evident manifestations of intoxication were as follows: there was a latent period following injection and lasting for an hour or longer, during which the animals appeared to be normal. They then became less active and were disinclined to feed. Generalized weakness increased and during the next few hours the chicks became ataxic and finally unable to stand. Hyperthermia appeared after two or three hours. Respiratory distress, indicated by rapid and labored breathing, was noticeable. There was a profuse fluid diarrhea. In the terminal stages the chicks became completely immobilized and unresponsive to stimuli. Death occurred

TABLE 4

CHICK LD_{50} OF SALMONELLA PULLORUM

Inoculum	Observed		Accumulated		Per Cent
	Deaths	Survivors	Deaths	Survivors	Mortality
10^5	12	0	43	0	100
10^4	12	0	31	0	100
10^3	12	0	19	0	100
10^2	5	7	7	7	50
10^1	2	10	2	17	10.5
10^0	0	12	0	29	0.0

Calculated LD_{50} $10^{2.4}$ cells (Reed and Muench, 1938).
Abridged table of results.

TABLE 5

CHICK LD_{50} OF THE ENDOTOXIN OF SALMONELLA PULLORUM

Inoculum	Observed		Accumulated		Per Cent Mortality
	Deaths	Survivors	Deaths	Survivors	
12.5	12	0	36	0	100
10.0	10	2	24	2	92.5
7.5	6	6	14	8	63.5
5.0	8	4	8	12	40.0
2.5	0	12	0	24	0.0

Calculated LD_{50} 6.4 mg (Reed and Muench, 1938).
Abridged table of results.

after periods of four to forty hours, depending on the size of the dose of endotoxin.

At autopsy the chicks showed extensive subcutaneous hemorrhaging, indicating profound vascular disturbances. Small areas of acute cellular necrosis occurred in the liver, spleen, and mycocardium. Small thrombi attached to the veins of the liver and other organs could be seen. The intestine was hemorrhagic and distended with a yellow fluid. The liver was edematous and showed evidence of fatty infiltration. Kidneys were hemorrhagic, with evidence of necrosis. Lungs were congested and hemorrhagic. The brain and adrenals were hemorrhagic.

Amino Acid Profiles in the Blood and Liver

Chromatographic analyses of the free and bound amino acids of the blood and of the total amino acids of the liver were made at intervals of six, twelve, thirty-six, forty-eight, sixty, and seventy-two hours in the following experimental groups: (1) chicks inoculated with 6.0 mg of endotoxin, (2) chicks treated with 6.0 mg of endotoxin and inoculated with glycine, (3) chicks infected with 10^3 viable cells of *S. pullorum*, (4) chicks infected with 10^3 viable cells of *S. pullorum* and treated with glycine, (5) glycine treated controls, and (6) normal chicks (Table 6).

Typical chromatograms prepared from the experimental groups at twenty-four hours are presented in Figures 3, 4, and 5. Sixteen amino acids were identified on the sheets prepared in the study of the free amino acids of the blood. Fifteen amino acids appeared on the

TABLE 6

EFFECT OF THE ADMINISTRATION OF AMINO ACIDS ON THE SURVIVAL TIME OF CHICKS INTOXICATED WITH THE ENDOTOXIN OF SALMONELLA PULLORUM

Group	Deaths/Survivors	Average Hour of Death
Endotoxin (6.0 mg)	19/16	30.2
Endotoxin and Glycine	21/14	33.4
Endotoxin and Arginine	9/26	45.0
Infected (10^3 cells)	35/0	59.2
Infected and Glycine	35/0	64.3
Normals	0/35	-
Glycine controls	0/35	-

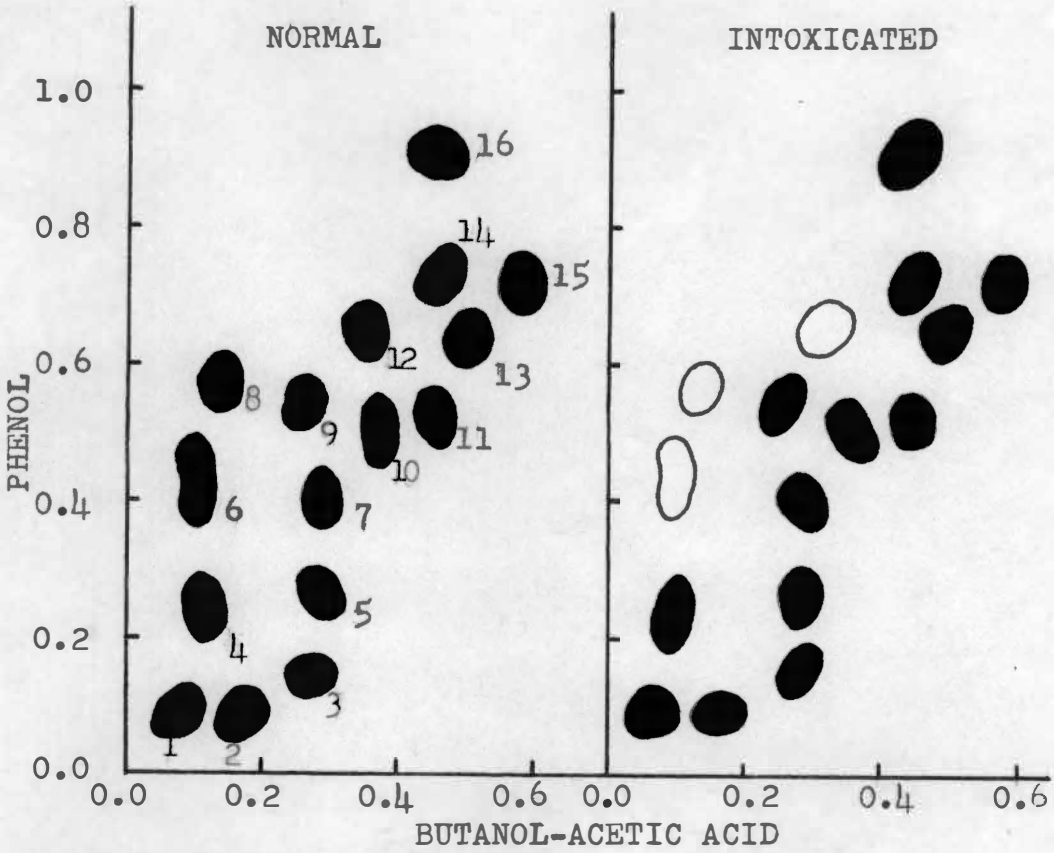

Figure 3. Typical chromatograms of the free amino acids in the blood of normal and intoxicated chicks. Spots are identified as follows: 1. cystine, 2. aspartic acid, 3. glutamic acid, 4. cysteine, 5. serine, 6. glycine, 7. threonine, 8. arginine, 9. alamine, 10. tyrosine, 11. hydroxyproline, 12, methionine, 13. valine, 14. phenylalanine, 15. tryptophan, and 16. proline.

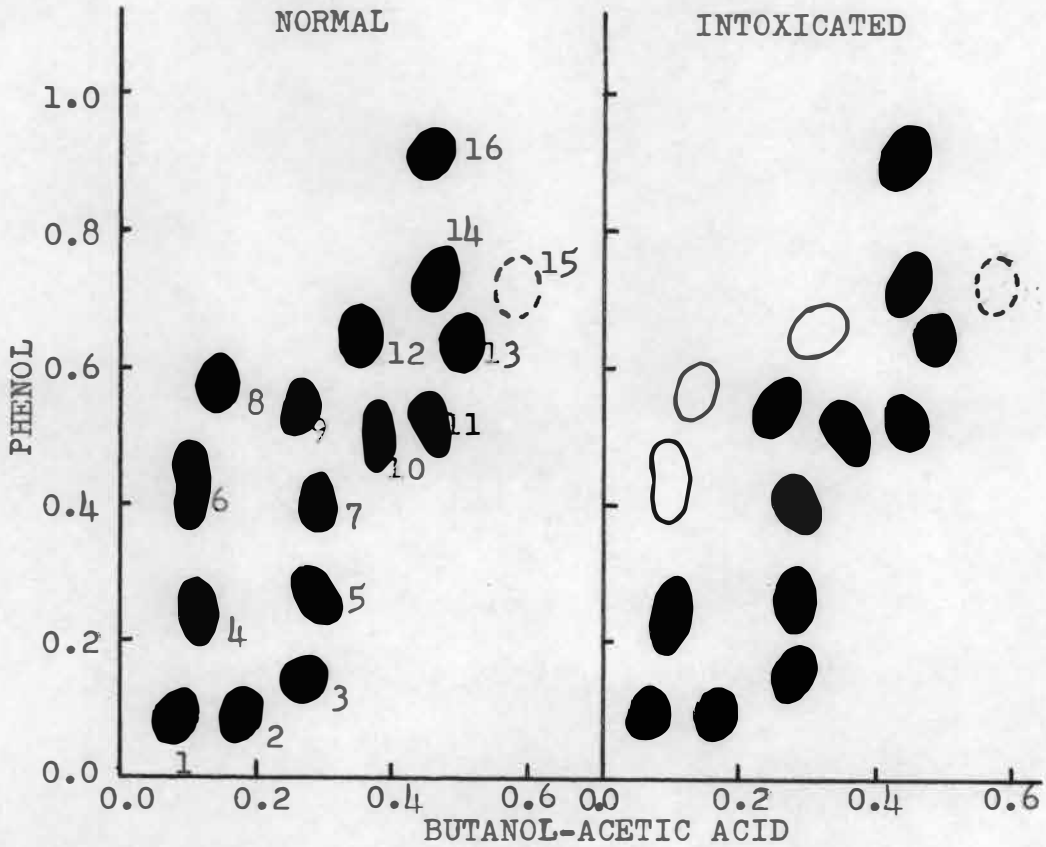

Figure 4. Typical chromatograms of the bound amino acids of the blood of normal and intoxicated chicks. Spots are identified as follows: 1. cystine, 2. aspartic acid, 3. glutamic acid, 4. cysteine, 5. serine, 6. glycine, 7. threonine, 8. arginine, 9. alanine, 10. tyrosine, 11. hydroxyproline, 12. methionine, 13. valine, 14. phenylalanine, 15. tryptophan-absent, and 16. proline.

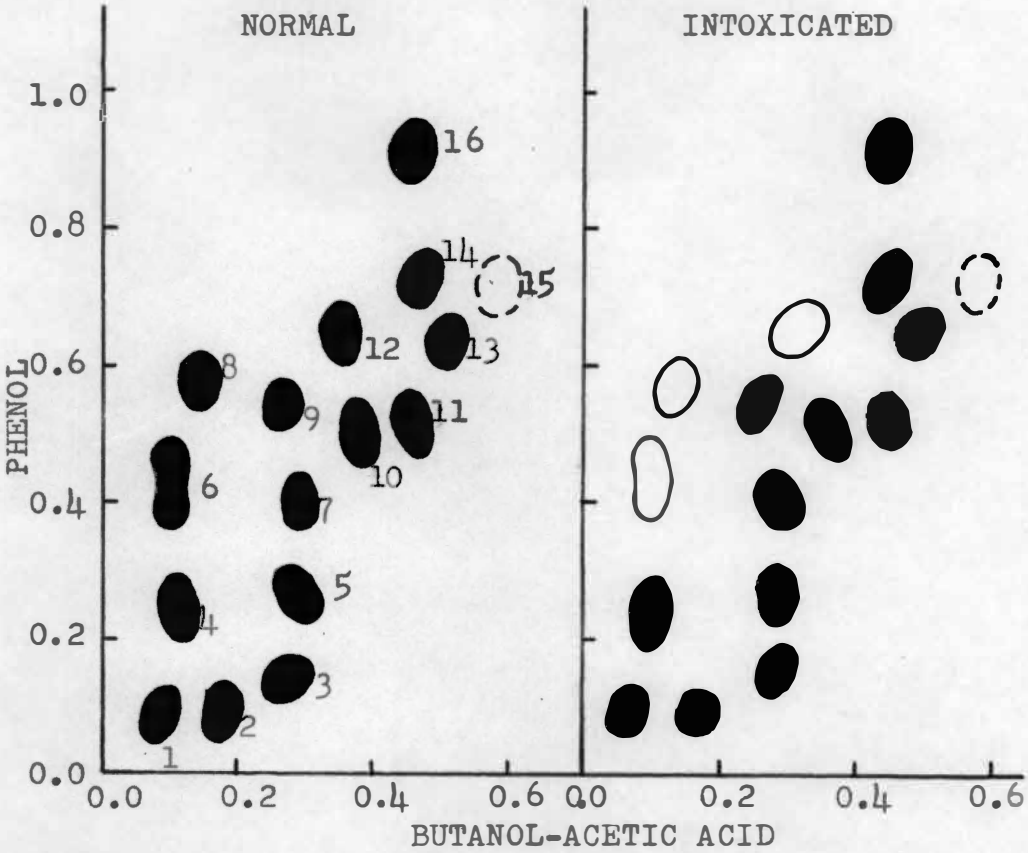

Figure 5. Typical chromatograms of the total amino acids (free and bound) of the livers of normal and intoxicated chicks. Spots are identified as follows: 1. cystine, 2. aspartic acid, 3. glutamic acid, 4. cysteine, 5. serine, 6. glycine, 7. threonine, 8. arginine, 9. alanine, 10. tyrosine, 11. hydroxyproline, 12, methionine, 13. valine, 14. phenylalanine, 15. tryptophan-absent, and 16. proline.

chromatograms prepared from the serum hydrolysates and liver tissue hydrolysates. Tryptophan, known to be destroyed by acid hydrolysis, was absent from the latter chromatographic sheets.

A complete graphical presentation of the changes occurring in the amino acid profiles of the blood and liver was not considered to be essential for an understanding of the effects of intoxication and infection. Such a presentation has not been given. Instead, the results concerned with these essential amino acids found to be altered by intoxication and infection have been discussed and compared. For convenience, chicks receiving injections of S. pullorum organisms have been called "infected," while animals receiving injections of endotoxin have been designated as "intoxicated" chicks in the following discussion.

Three essential amino acids were found to be reduced in concentration in the blood and liver of intoxicated and infected animals. These were arginine, glycine, and methionine. The concentration profiles of these amino acids will be discussed on an individual basis.

In normal untreated chicks arginine, glycine, methionine, and tryptophan were identified on all chromatograms of the free amino acids of the blood at all time intervals studied. Tryptophan could not be identified on the chromatograms of the bound amino acids of the blood serum and liver tissue of normal chicks. Attempts to selectively eliminate arginine, glycine, and methionine from these chromatograms by dilution of the filtrates or hydrolysates were

unsuccessful. Normal animals receiving injections of one ml of sterile physiological saline presented amino acid profiles similar to those of untreated normal animals.

Profiles of arginine in intoxicated and infected chicks. Intoxicated chicks showed a reduction in the concentration of arginine in the blood and liver. The reduction was detectable at six hours and became marked at twelve hours. Using the dilution procedure, it became possible to selectively eliminate arginine from chromatograms prepared at twenty-four hours. The concentration of arginine in the hydrolysates of the serum proteins and liver was reduced at twelve hours and continued to decline until death or until the animals began to recover. In survivors of sublethal doses of endotoxin, normal concentrations of arginine were re-established, in most cases, between seventy-two and ninety hours. The administration of glycine did not appear to affect the concentrations of arginine in intoxicated animals. The administration of arginine, however, appeared to be effective in retaining of normal concentrations of this amino acid in the blood and liver of intoxicated chicks.

In the case of chicks infected with virulent *S. pullorum* the concentration of free arginine in the blood was reduced at six hours and became markedly reduced between twelve and twenty-four hours. The level of bound arginine in the serum proteins and liver tissue became detectably decreased at twenty-four hours. Between thirty-six and forty-eight hours the spot identified as arginine could be selectively eliminated from the chromatographic sheets by the dilution

procedure. At sixty hours the spot for arginine could be detected only if 0.5 ml of the filtrates were applied to the sheets. Since most of the infected animals were either dead or prostrate at seventy-two hours, only a few analyses were made. These seventy-two hour chromatograms, however, revealed marked reductions in the concentration of arginine in the blood and liver.

The administration of glycine to infected chicks did not appear to exert any sparing effect on arginine concentrations in these animals although survival times were slightly extended (Table 6). The administration of arginine, however, appeared to aid in the retention of the concentration of this amino acid at near normal levels in the blood, especially during the period between six and twenty-four hours. Also, the administration of arginine extended the survival time of infected chicks.

Profiles of glycine in intoxicated and infected chicks. Chicks intoxicated with endotoxin showed a marked reduction in the concentration of free glycine in the blood which became detectable, in some cases, at twelve hours. At twenty-four hours the decreases were evident in all animals. The reductions in the concentration of glycine in the serum proteins and liver, however, were not detectable, in most cases, until approximately thirty-six hours. When compared to the reductions observed in the case of arginine, the decreases in glycine concentration appeared to be rather gradual and uniform. In the cases of animals surviving sublethal doses of endotoxin, the lowest concentrations of glycine were observed between thirty-six and sixty hours. Increased

concentrations of glycine were noted as survivors began to take food.

The administration of glycine to intoxicated chicks appeared to aid in the maintenance of normal or near normal concentrations of the amino acid in the blood during the early stages of the experimental period. Survival time was slightly extended by the injection of glycine (Table 6).

In the case of infected chicks, the reductions in the concentrations of free glycine in the blood became detectable at twelve hours in most animals. The decrease became evident in all animals at twenty-four hours. In the serum proteins and liver the reduction in the concentration of glycine became detectable between twenty-four and thirty-six hours. The declines in the concentration of glycine appeared to be rather gradual and uniform when compared to the arginine response in infected chicks.

In the early stages of infection, the administration of glycine appeared to aid in the maintenance of near normal levels of the amino acid in the blood. In the terminal phases, however, administration of glycine did not appear to alter the amino acid response, although survival times were slightly extended by the treatment (Table 6).

Profiles of methionine in intoxicated and infected chicks. In chicks intoxicated with endotoxin the decline in the concentration of methionine in the blood and liver became detectable by the dilution procedure at twenty-four hours. The reductions occurring between twenty-four and thirty-six hours were rather marked in the case of free methionine in the blood. The lowest concentrations of methionine

in the serum proteins and liver tissue were observed at approximately sixty hours. The administration of arginine appeared to accelerate the reduction of both methionine and glycine from the blood. The administration of glycine, however, did not appear to alter the response of methionine in intoxication.

In the case of chicks receiving injections of S. pullorum organisms, the decrease in the concentration of free methionine in the blood was gradual and rather uniform. Lowest concentrations were detectable between forty-eight and sixty hours. The decline of methionine in the serum proteins and liver tissue became evident at approximately forty-eight hours. The administration of glycine did not appear to alter the response of methionine in infected chicks. The administration of arginine, however, appeared to accelerate the disappearance of both glycine and methionine from the blood during the latter stage of the experimental period.

Profiles of tryptophan in intoxicated and infected chicks. Tryptophan concentrations in the protein-free filtrates of the blood did not appear to be altered during intoxication with endotoxin. This amino acid was detectable on chromatograms prepared from these filtrates at all time intervals.

Acid hydrolysis, however, is known to destroy tryptophan, and under the conditions of these determinations one would not expect to find this amino acid on sheets prepared from the hydrolysates of the serum proteins and liver tissue (Figures 4 and 5).

The concentration of free tryptophan in the blood of chicks

infected with S. pullorum did not appear to be altered.

Effect of Endotoxin on the Body Temperature of Chicks

The body temperature response of chicks receiving injections of 0.5 mg of the endotoxin of S. pullorum was studied (Figure 6). The effect of the administration of cortisone on the body temperature of intoxicated chicks was also observed (Table 7). The results confirmed the pyrogenic nature of the endotoxin of S. pullorum. Cortisone was also found to alter the response of the chick to the pyrogenic endotoxin by preventing the body temperature from increasing.

Changes in the Blood Chemistry of Chicks During Intoxication and Infection

The results of the blood chemistry studies and determinations are presented in Tables 8, 9, and 10. The nitrogen containing compounds of the blood including urea, creatinine, uric acid, and ammonia were found to be increased during intoxication. The alterations were found to be very similar, in most cases, to those observed in chicks infected with S. pullorum.

Blood sugar was decreased and non-protein nitrogen was increased at twenty-four hours in the intoxicated chicks. Inorganic phosphorus of the serum and the hemoglobin concentration were reduced in both the intoxicated and infected chicks at twenty-four hours.

The administration of cortisone to the intoxicated chicks appeared to increase the concentrations of non-protein nitrogen, uric acid, and glucose in the blood. Treatment with the hormone, however,

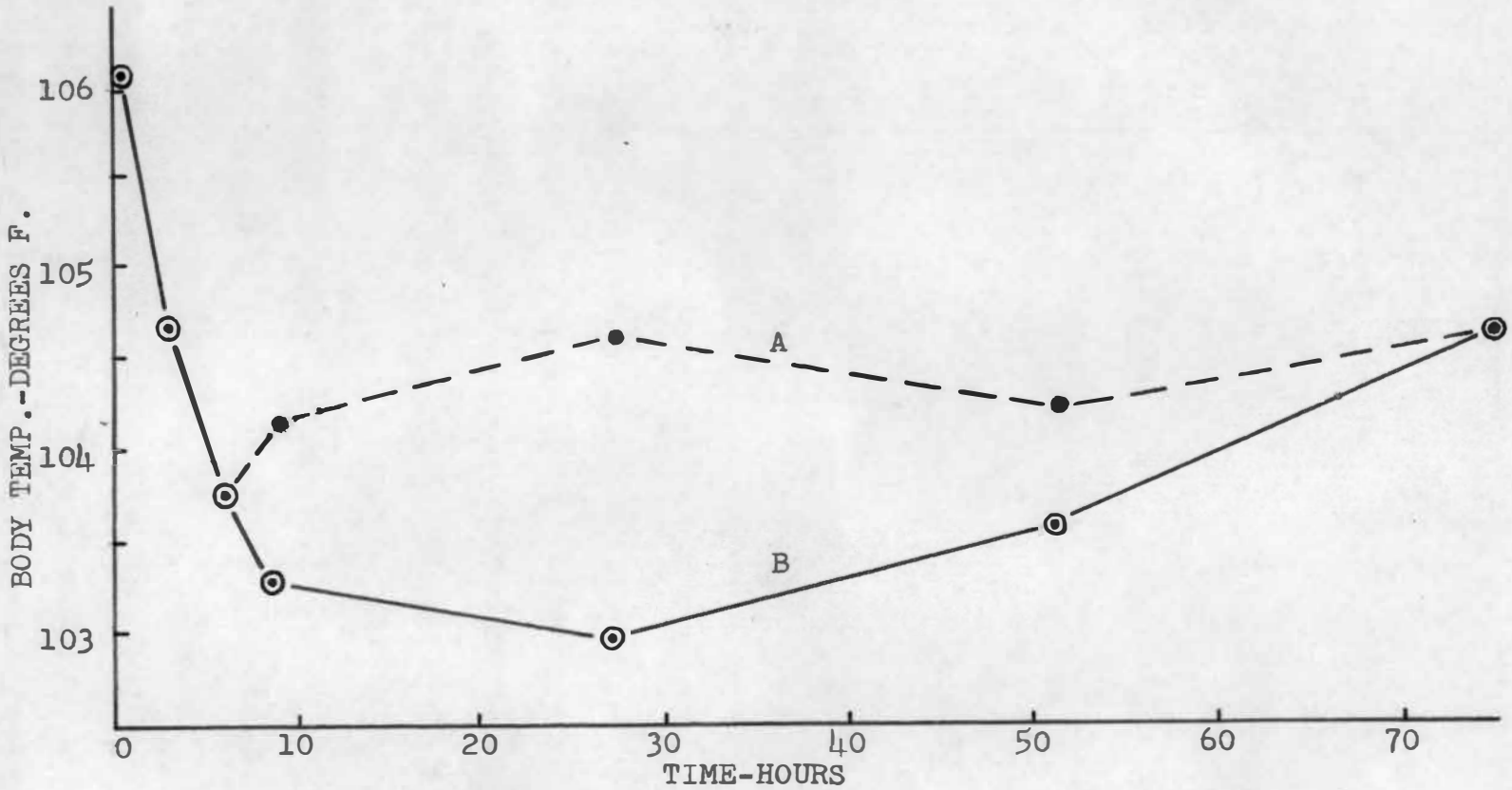

Figure 6. Body temperature of chicks inoculated with 0.5 mg of *Salmonella pullorum* endotoxin. Line A intoxicated chicks. Line B normal untreated chicks.

TABLE 7

EFFECT OF CORTISONE ON THE BODY TEMPERATURE (F.) OF CHICKS INOCULATED WITH 0.5 MG OF THE ENDOTOXIN OF SALMONELLA PULLORUM

Time	Endotoxin (0.5 mg)	Endotoxin and Cortisone	Normal Controls	Saline Controls
0	103.7	103.7	103.7	103.7
8	104.1	103.5	103.3	103.7
12	104.2	103.5	103.2	103.6
24	104.6	103.8	103.0	103.3
36	104.5	103.3	103.2	103.5
48	104.3	103.7	103.5	103.7
60	104.4	103.8	104.0	104.0
72	104.6	104.1	104.4	104.5

Chicks injected with cortisone twelve hours prior to the start of the experiment and twelve hours following the injection of endotoxin.

TABLE 8

NON-PROTEIN NITROGEN, UREA AND CREATINE IN THE BLOOD OF CHICKS INTOXICATED WITH THE ENDOTOXIN OF *SALMONELLA PULLORUM* AND INFECTED WITH *SALMONELLA PULLORUM**

Group	NPN mg Per Cent	Per Cent Normal	Urea-N mg Per Cent	Per Cent Normal	Creatine mg Per Cent	Per Cent Normal
Normal	33.8	100	0.33	100	0.70	100
Endotoxin	37.5	111	2.28	690	1.27	182
Endotoxin and Cortisone	41.8	124	2.29	693	0.92	130
Infected	39.7	118	2.11	642	2.08	298
Infected and Cortisone	42.0	125	2.45	743	2.23	318
Cortisone Controls	37.5	111	0.37	114	0.80	114

*Averages of four determinations on pooled samples from three chicks each taken at twenty-four hours.

TABLE 9

URIC ACID, AMMONIA AND HEMOGLOBIN CONCENTRATION IN THE BLOOD OF CHICKS INTOXICATED WITH THE ENDOTOXIN OF *SALMONELLA PULLORUM* AND INFECTED WITH *SALMONELLA PULLORUM**

Group	Uric Acid mg Per Cent	Per Cent Normal	NH_3-N uM/ml	Per Cent Normal	Hemoglobin mg Per Cent	Per Cent Normal
Normal	3.18	100	0.67	100	7.60	100
Endotoxin	4.25	134	1.01	150	6.10	80
Endotoxin and Cortisone	4.31	136	0.70	104	5.70	75
Infected	4.14	131	0.94	130	6.00	79
Infected and Cortisone	4.50	142	1.25	209	6.00	79
Cortisone Controls	3.20	102	0.73	108	7.40	98

*Averages of four determinations on pooled samples from three chicks each taken at twenty-four hours.

TABLE 10

BLOOD SUGAR AND INORGANIC PHOSPHORUS CONCENTRATIONS OF CHICKS INTOXICATED WITH THE ENDOTOXIN OF *SALMONELLA PULLORUM* AND INFECTED WITH *SALMONELLA PULLORUM**

Group	Blood Sugar mg Per Cent	Per Cent Normal	Inorganic-P mg Per Cent	Per Cent Normal
Normal	195.4	100	3.43	100
Endotoxin	155.6	80	4.10	120
Endotoxin and Cortisone	177.1	91	4.00	118
Infected	163.4	83	4.20	124
Infected and Cortisone	135.8	69	4.40	130
Cortisone Controls	188.9	98	3.53	103

*Averages of four determinations on pooled samples from three chicks taken at twenty-four hours.

appeared to reduce the concentrations of blood ammonia in the intoxicated animals. The concentrations of urea, inorganic phosphorus and glucose in the blood did not appear to be significantly altered by cortisone treatment under the conditions of this experiment.

In the case of chicks infected with S. pullorum the administration of cortisone had the effect of completely disorganizing the host-parasite relationship. The infectious process appeared to be enhanced and accelerated by the administration, although outwardly the treated animals appeared to be normal until shortly before death. At twenty-four hours many of the animals in the group receiving cortisone were dead. The cortisone treated, infected chicks demonstrated a marked hypoglycemia at twenty-four hours (Table 10) indicating the rapid course of the disease. Increases in the inorganic phosphorus of the serum and the nitrogen containing compounds also indicated that infectious process was markedly accelerated by cortisone (Tables 8, 9, and 10). The concentration of hemoglobin in the infected chicks did not appear to be effected by cortisone (Table 9).

The effect of the administration glycine on the hemoglobin concentration of intoxicated chicks was studied and the results are presented in Figure 7. The injection of this amino acid, under the conditions of this experiment, appeared to exert a sparing effect on the hemoglobin concentrations of intoxicated chicks between twenty-four and sixty hours.

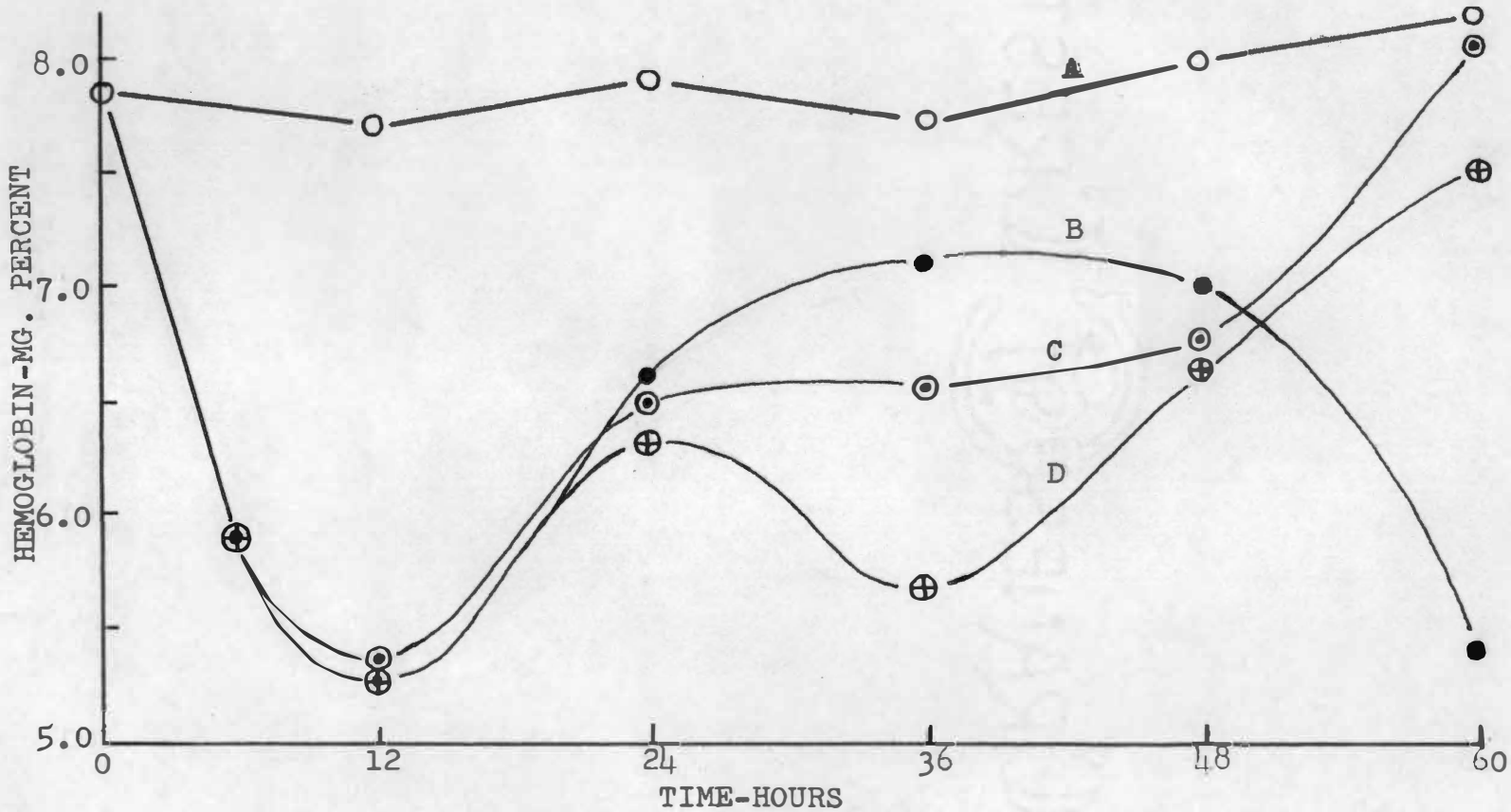

Figure 7. Effect of the administration of endotoxin on the hemoglobin concentrations of chicks intoxicated with Salmonella pullorum endotoxin. Line A normal chicks. Line B chicks infected with S. pullorum. Line C intoxicated chicks treated with glycine. Line D intoxicated, untreated chicks.

Concentrations of Citrate in the Livers of Intoxicated and Infected Chicks

The results of the citrate determinations in the livers of normal, intoxicated, and infected chicks are presented in Table 11. At twenty-four hours following the injection of endotoxin or infection with *S. pullorum* the concentration of citrate in the liver increased.

Coenzyme A Levels in the Livers of Intoxicated and Infected Chicks

Coenzyme A levels in the livers of normal, intoxicated, and infected chicks were determined and the results are presented in Table 11. Under the conditions of this experiment the coenzyme A levels appeared to be reduced in the livers of both the intoxicated and infected animals.

TABLE 11

COENZYME A AND CITRATE LEVELS IN THE LIVERS OF CHICKS INTOXICATED WITH THE ENDOTOXIN OF *S. PULLORUM* OR INFECTED WITH *S. PULLORUM* TAKEN AT TWENTY-FOUR HOURS

Group	Citrate ug/g Wet Tissue	Pantothenate ug/g Wet Tissue	Coenzyme A Units*
Normal	96.1	150.2	214.8
Endotoxin	110.6	126.3	180.6
Infected	108.5	133.7	191.2

*Coenzyme A units calculated (Olson and Kaplan, 1948).

TABLE 12

BLOOD AMMONIA LEVELS IN CHICKS INTOXICATED WITH THE ENDOTOXIN OF *S. PULLORUM* OR INFECTED WITH *S. PULLORUM* AT THIRTY-SIX HOURS

Group	Blood Ammonia - uM/ml	
	Portal Vein	Caudal Vena Cava
Normal	1.10	0.67
Endotoxin (6.0 mg)	1.28	1.01
Endotoxin and Cortisone	0.95	0.70
Infected (10^3 cells)	1.32	0.94
Infection and Cortisone	1.41	1.25
Cortisone Controls	1.00	0.73

TABLE 13

THE EFFECT OF CORTISONE ON THE SURVIVAL TIME OF CHICKS INTOXICATED WITH THE ENDOTOXIN OF S. PULLORUM OR INFECTED WITH S. PULLORUM

Group	Deaths/Survivors	Average Hour of Death
Infected (10^3 cells)	35/0	59.2
Infected and Cortisone	10/0	31.6
Endotoxin	19/16	30.2
Endotoxin and Cortisone	3/7	43.0
Cortisone Controls	0/10	--

TABLE 14

EFFECT OF THE ADMINISTRATION OF ARGININE ON THE BLOOD AMMONIA AND UREA LEVELS OF CHICKS INTOXICATED WITH THE ENDOTOXIN OF S. PULLORUM AND INFECTED WITH S. PULLORUM AT TWENTY-FOUR HOURS

Group	Blood NH_3-N Caudal Vena Cava uM/ml	Blood Urea-N mg Per Cent
Normal	0.67	0.33
Endotoxin (6.0 mg)	1.01	2.28
Endotoxin and Arginine	0.73	2.29
Infected (10^3 cells)	0.94	2.11
Infected and Arginine	0.77	2.25
Arginine Controls	0.75	0.41

CHAPTER V

DISCUSSION

When first isolated by Boivin *et al.* (1933) the somatic antigens or endotoxins of the gram-negative bacteria were considered to be lipopolysaccharides. Subsequently, Freeman *et al.* (1940) and Anderson (1941) reported that the somatic antigens were phosphorus containing protein-polysaccharide-lipid complexes. The investigations of Partridge and Morgan (1942) indicated that the lipid could be separated from the complex, but that the protein and polysaccharide were essential for toxic and antigenic functions. Webster *et al.* (1955), however, showed that the somatic antigen of *S. typhosa* could be obtained in a form essentially free of protein. This preparation exhibited the complete array of properties associated with the endotoxins of gram-negative bacteria.

The presence of protein, polysaccharide and lipid moieties in the endotoxin of *S. pullorum* has been confirmed during this investigation by infrared absorption obtained from two different spectrometers and techniques. The following points on the spectra were considered to be significant:

3.43 microns: The band at 3.43 microns, indicative of the presence of methyl groups, appeared to be rather intense in the endotoxin.

3.53 microns: A small but significant peak was found on the spectra at this point. These peaks may not be readily discernible in

Figures 1 and 2 due to the reduction necessary for reproduction of the spectra.

5.80 microns: This peak, due to the carbonyl of the carboxyl group of the lipid moiety, was relatively strong in intensity in the molecule. The peak is overlapped by another peak at 6.05 microns which tends to mask its intensity.

6.05 microns: This peak which is attributed to the carbonyl of the peptide linkage is indicative of the presence of protein. When evaluated with the bands at 3.0 and 6.0 microns, this peak is considered to be conclusive evidence for the presence of protein in the molecule.

6.50 microns: The peak at this wavelength is attributed to the N-H deformation as found in the peptide linkage. When considered with the peaks at 3.0, 6.0, and 6.05 microns, this peak is strong evidence for the presence of protein in the endotoxin.

9.0-10.0 microns: The peaks between 9.0 and 10.0 microns are generally characteristic of polysaccharide. This broad band absorption confirmed the presence of large amounts of polysaccharide in the endotoxin. Neucleic acids which may also absorb in this region were shown to be absent by ultra-violet absorption studies.

In summary, the infrared spectra from two different spectrometers indicated that the endotoxin of *S. pullorum* contained protein, polysaccharide, and lipid moieties. The general appearance of the spectra also indicated that the endotoxin of *S. pullorum* was closely related and similar to endotoxins obtained by other investigators from

a variety of gram-negative microorganisms.

The amino acids involved in the protein moiety and the sugars composing the polysaccharide fraction of the endotoxin of S. pullorum CDC 3522/51 have not previously been described. Salton (1953), in a study of the composition of the cell walls of a number of bacteria, found fifteen amino acids in the hydrolysates of the cell walls of S. pullorum. Galactose, glucose, mannose, rhamnose, and hexosamine were found to be involved in the cell wall structure. The results of this investigation (Table 3) are in agreement with the findings of Salton (1953) and this agreement serves to emphasize the close relationship of the somatic antigen or endotoxin and the cell wall structure of S. pullorum.

The LD_{50} determinations indicated that the chick is a rather resistant animal, on a body weight basis, to the lethal action of pullorum endotoxin. Burrows (1951) reported that mice were relatively uniformly susceptible to doses of 0.1 to 0.5 mg of purified endotoxin. The rat was found to be more resistant and the median lethal dose was 6.0 to 8.0 mg. The apparent resistance of the chicks to the lethal action of endotoxin could help explain the inability of Hanks and Rettger (1932) to kill chicks with their preparation. It is unlikely that the endotoxin was present in their cultural filtrates in sufficient concentrations to be lethal for chicks. The filtrates, however, proved to be lethal for the more susceptible animals including rabbits and guinea pigs.

The LD_{50} for the experimental strain of S. pullorum was in

agreement with the values reported by other investigators (Ross et al., 1955a; Gilfillan et al., 1956). The average hour of death for chicks injected with 10^3 viable organisms was 59.2 (Table 3).

The resemblance between the manifestations of endotoxin intoxication and traumatic shock are emphasized by the results of this investigation. The vascular disturbances following the injection of endotoxin are comparable with those observed in various types of shock and induced stress. The metabolic alterations following the injection of endotoxin including hyperglycemia, early disappearance of liver glycogen, elevated levels of lactate and pyruvate in the blood, and increases in the inorganic phosphorus in the blood are changes which also occur in shock (Thomas, 1954). Fever, although more common in the endotoxic reaction, may also occur in shock. Gastrointestinal miral hemorrhages occur in shock and intoxication (Delaunay et al., 1948). Nitrogen metabolism is also altered during shock and the nitrogen balance becomes markedly negative (Schenker, 1939; Albright, 1943; Glendening et al., 1944). The typical symptoms of endotoxic shock have been observed in humans following transfusion with contaminated blood (Borden and Hall, 1951).

The consistent effect of endotoxin on the blood sugar of the chick was suggestive of an impairment of carbohydrate metabolism. The basis for the changes in the blood sugar accompanying intoxication or infection with S. pullorum is not known. It has been shown that the glycogen reserves of the intoxicated animal become depleted by the time the hypoglycemia reaches maximum levels (Kun and Miller, 1948;

Kun, 1948a; Kun and Abood, 1949). The hyperglycemia has been attributed to a central stimulation of the adrenals by way of the splanchnic nerve with consequent glycogenolysis (Evans and Zwecker, 1927). Lawrence and Buckley (1927) thought that both the adrenals and thyroid were involved in the hyperglycemia following the injection of diphtheria toxin. Soskin et al. (1935) expressed the idea that the abnormal glucose tolerance curves obtained following the injection of a toxin were due to the effects of the compound on the liver and not on the pancreas. The result was an interference with the homeostatic mechanism of the liver. Kun (1948b) showed that the utilization of glucose by muscle extracts from rats injected with endotoxin was inhibited in vitro. This inhibition was neutralized by insulin. It was concluded, therefore, that the activity of hexokinase was reduced by endotoxin. The observations of Kun and his associates help to explain, more completely, in earlier observations of Soskin et al. (1935).

It appears that the hypoglycemia which follows the hyperglycemia may be attributed to the combined action of several factors. The hyperthermia and inanition, with the implied accelerated metabolic rate, could result in the increased utilization of glucose during a period of reduced intake. Also, the faulty utilization of glucose could prevent the proper conservation of carbohydrate reserves during intoxication.

The accumulation of citrate in the livers of chicks treated with the endotoxin of S. pullorum revealed a biochemical disturbance in these animals not previously reported. This accumulation is

evidence of a block in the tricarboxylic acid cycle and it confirms the earlier observations of disturbances in the carbohydrate metabolism attributable to endotoxins. The accumulation of citrate in the livers of chicks treated with pullorum endotoxin is also of interest when compared to the results obtained by Berry et al. (1954b) and by Gilfillan et al. (1956b) in experiments with inhibitors of the tricarboxylic acid cycle. These investigators found that citrate accumulated in mice receiving injections of large quantities of heat-killed S. typhimurium cells and in the livers of chicks infected with S. pullorum. It appeared that the processes leading to the death of the animals in these experiments were closely related through the common biochemical alterations produced by the endotoxins of these organisms. The work of Kun and Miller (1948) could serve to help explain the accumulation of citrate in intoxicated animals. These investigators found that endotoxin markedly inhibited the activity of succinic dehydrogenase in the muscle and liver of rabbits. The inhibition of this terminal oxidative system would be expected to lead to an accumulation of citrate. Much work remains to be done, however, before the complete picture of the mode of action of endotoxin emerges. There appears to be, nevertheless, a relationship between the biochemical effect of endotoxin and the mode of action of some of the inhibitors of the tricarboxylic acid cycle.

The apparent reduction in the coenzyme A levels in the livers of chicks intoxicated with pullorum endotoxin (Table 10) could help explain more completely the accumulation of lactate in the blood and

tissues of intoxicated animals. The reduction of coenzyme A, by preventing the entry of pyruvate into the tricarboxylic acid cycle, could contribute to the induction of a state of tissue anoxia. Under these conditions lactate could be expected to accumulate. The work of Kun and Abood (1949) showed that the activity of pyruvic oxidase was markedly inhibited by endotoxin in rabbit muscle and liver. Thus, the accumulation of lactic acid might be attributed to the inhibition of the condensation reaction to form oxalacetate from pyruvate.

Whether the observed reductions of coenzyme A levels are due to destruction, inactivation or inhibition of synthesis remains to be determined. The synthesis of coenzyme A is known to be dependent upon the availability of adenosine triphosphate (ATP) and the apparent reduction could be the result of the inhibition oxidative phosphorylation by endotoxin. Additional evidence for this idea was recently obtained by Takeda et al. (1955). These workers observed that the administration of ATP with endotoxin reversed lethal effect of the toxin, including the accumulation of lactic acid, increases in inorganic phosphorus and alpha-ketoglutarate in the blood and the decrease of organic phosphorus of the blood.

It is of interest at this point to note that a decrease in coenzyme A is a characteristic of fatty livers (Severi and Fonnesu, 1956). The reduction of coenzyme A could be a contributing factor in the pathological accumulation of fat in the livers of intoxicated animals.

The observations of alterations in the amino acid metabolism

and nitrogen excretion pattern of chicks treated with endotoxin represented a previously unreported biochemical disturbance attributable to gram-negative bacterial toxins. These findings are of interest when compared to those of Ross et al. (1956). The similarities of the responses of the chick to intoxication with endotoxin and infection with S. pullorum are striking and serve to emphasize the role of endotoxin in the pathology of pullorum disease in chicks. Both experimental treatments have been shown to reduce the concentration of arginine, glycine, and methionine in the blood and liver of the chick. Accompanying this reduction in the concentrations of three essential amino acids, changes in the concentrations of the nitrogen containing compounds of the blood have been demonstrated. Urea, uric acid, creatine, and ammonia have been shown to be increased both during intoxication and infection. During experimental intoxication and infection urea becomes the major nitrogen containing product in the blood of normally uricotelic animals.

The protective effect of arginine, based on the increased survival time of experimental animals (Table 6), has been demonstrated both in experimental intoxication and infection. Arginine has been shown to protect animals against the toxic effects of elevated levels of ammonia in the peripheral blood (Najarian and Harper, 1956; du Ruisseau et al., 1956). Normally, the large amounts of ammonia present in the portal blood are removed and metabolized by the liver. However, in the case of chicks intoxicated with endotoxin or infected with S. pullorum the ability of the liver to remove ammonia from the portal

blood appears to be impaired (Table 12). High levels of ammonia were found in the blood of intoxicated and infected chicks. The toxicity of high levels of ammonia in the peripheral circulation is well known (Bessman, 1956), and this factor could contribute to the lethal effect of pullorum disease.

In the course of this investigation the results indicated that the administration of endotoxin or infection with S. pullorum was accompanied by the activation of a hepatic system in the chick designed to cope with the increased blood ammonia coming to the liver in the portal blood. The administration of arginine appeared to accelerate and augment this hepatic system by permitting blood ammonia to be converted into urea and creatine. The resulting increased ability of the liver to cope with a larger amount of ammonia appeared to result in increased survival of intoxicated chicks (Table 6). A proposed scheme for this hepatic system is presented in Figure 8.

The protective effect of arginine appeared to be exerted through the Krebs "ornithine cycle" by a conversion of blood ammonia into arginine. Arginine could then serve as a precursor of urea or it could participate with glycine and methionine in the synthesis of creatine. The operation of the "ornithine cycle," however, depends on the presence of the enzyme arginase. This enzyme is not detectable in the liver of newly hatched chicks (Clementi, 1914, 1946; Ross et al., 1956). Arginase activity has been demonstrated, however in embryonated eggs from the second until approximately the twelfth day

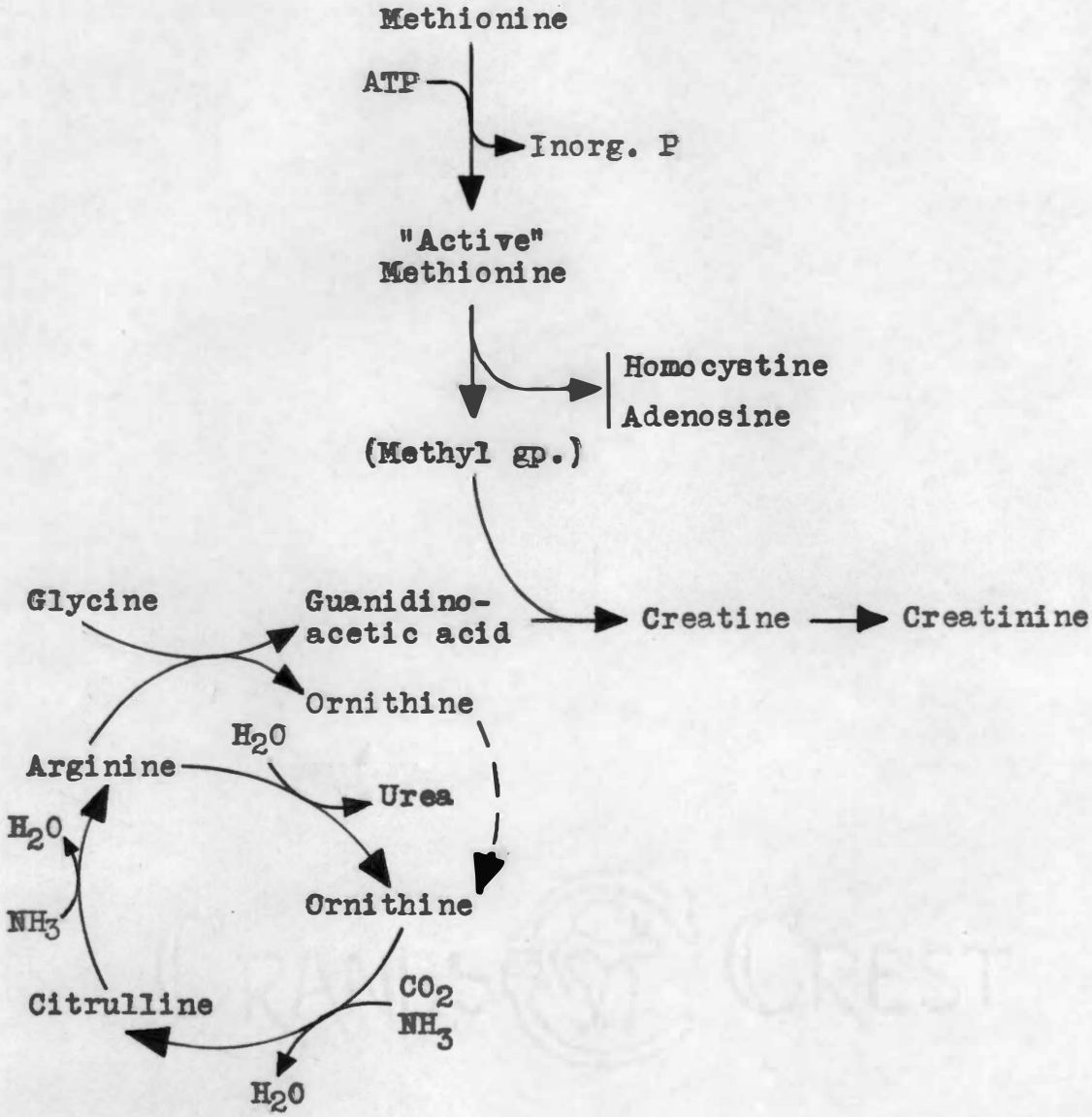

Figure 8. A proposed hepatic scheme relating amino acid metabolism and nitrogen excretion in chicks intoxicated with Salmonella pullorum endotoxin.

(Needham et al., 1935). From the twelfth day onward the activity of arginase is at a minimum and at hatching it is not demonstrable. Following the experimental infection of chicks with S. pullorum, Ross et al. (1956) demonstrated the presence of an active arginase system in the liver of these animals. The increased arginase activity could be correlated with the increase of urea in the blood and with the disappearance of arginine from the blood and livers of infected chicks. On the basis of their experimental results Ross and his associates attributed the protective effects of arginine to the activation of the "ornithine cycle" with the consequent production of urea. The administration of other reactants of the cycle including citrulline and ornithine also increased the survival time of infected chicks. Urea, in low concentrations, also extended the survival time of chicks infected with S. pullorum. Arginine, ornithine, or citrulline did not exert an inhibitory effect on the growth in vitro of the microorganisms. Urea, however, reduced the growth of S. pullorum in vitro in concentrations as low as 250 mM and was completely inhibitory at concentrations 1 M. The protective role of urea and its precursors in the "ornithine cycle" appeared to be related to some differential toxicity of urea for the chick and S. pullorum. The toxicity of urea, in sufficient concentration, for uricotelic animals is well known.

The protective role of arginine for chicks intoxicated with pullorum endotoxin, as observed during this investigation, also appeared to be related to the reactivation of the Krebs "ornithine cycle" in the liver. Arginine increased the survival time of

intoxicated chicks (Table 6). Since the decrease in concentration of arginine in the blood and liver was accompanied by a decrease in peripheral blood ammonia and an increase in blood urea and creatine (Tables 8 and 14), it appeared that these compounds were of arginolytic origin. The "ornithine cycle" might be expected to operate in the intoxicated chick until the concentration of urea in the blood reached toxic levels for the host or until damage in the liver prevented the continuation of the synthesis.

The source of the increased ammonia in the portal blood of the intoxicated chick is not definitely known. It is known, however, that bacteria capable of producing ammonia from the breakdown of amino acids or the splitting urea are present in the natural flora of the intestine of many animals. It appeared that the increased ammonia in the portal blood of the intoxicated chick could be the result of bacterial action. Phear and Ruebner (1956) found that the intestinal flora of patients with cirrhosis of the liver and demonstrating elevated peripheral blood ammonia levels differed from that of normal patients. The patients with cirrhosis showed greatly increased numbers of coliforms and Streptococcus faecalis in the small intestine. These organisms were found to occur at higher levels in the intestines of cirrhotics. The administration of antibiotics effective against the principal ammonia producers appeared to reduce the amount of ammonia in the blood of cirrhotics (Phear and Ruebner, 1956).

In the case of chicks intoxicated with endotoxin or infected with S. pullorum, changes in the intestinal flora could be related to

an occlusion of the bile duct. Such occlusions are a consistent feature in the pathology of pullorum disease and they are commonly observed in animals dying from the results of intoxication with pullorum endotoxin. The gall bladder becomes distended and the contents are viscid. The restriction or cessation of the flow of bile could lead to marked changes in the flora of the small intestine. Such a change in the flora might be responsible for the increased production of ammonia.

The metabolic conversion of glycine is known to include participation with arginine in a transamidination reaction by which the amidine group from arginine is transferred to the nitrogen of glycine to form guanidinoacetic acid (Figure 8). The reaction was demonstrated in the intact rat by Block and Sshoenheimer (1940) and in the presence of kidney slices by Dubnoff and Borsook (1941). Borsook and Dubnoff (1947) have shown that liver preparations can transfer the methyl group of methionine to guanidinoacetic acid, thereby forming creatine. The nitrogen of creatine cannot be used by the body as a source for protein synthesis. Thus, creatine may be considered as the endproduct of the metabolism of the metabolism of arginine, glycine, and methionine (Fruton and Simmons, 1953).

In the case of the chick intoxicated with pullorum endotoxin or infected with S. pullorum, the excretion of creatine as creatinine is increased (Table 8). The acceleration of the biosynthesis of this compound could represent a pathway for the conversion of blood ammonia into an excreatable nitrogen compound (Figure 8).

Glycine also serves as a precursor of uric acid (Sonne et al., 1949; Buchanan et al., 1949). Since uric acid represents the principal nitrogen excretion product of the normal chick, and since it is excreted in increased amounts by intoxicated and infected chicks (Table 9), the biosynthesis of this nitrogen containing compound could help to account for the glycine shown to disappear from the blood and liver of experimental animals.

In connection with the increased excretion of uric acid by chicks intoxicated with the endotoxin of S. pullorum and infected with S. pullorum, it is of interest to note that the ability of avian liver to synthesize uric acid from ammonia was observed early in the study of the nitrogen excretion of fowls. Edson and Krebs (1936) showed that pigeon liver slices, on incubation with ammonia, gave rise to appreciable quantities of hypoxanthine which in the presence of xanthine oxidase was oxidized to form uric acid. This biosynthetic pathway could represent another hepatic system capable of reducing the ammonia in the blood by converting it into uric acid.

The results of this investigation indicate that a decrease in the hemoglobin concentration of the chick is a result of intoxication with pullorum endotoxin and infection with S. pullorum (Table 9). The administration of glycine to the intoxicated chicks appeared to prevent, at least in part, the marked reduction in hemoglobin between twenty-four and sixty hours (Figure 7). The utilization of glycine and acetate in the synthesis of heme has demonstrated not only in vivo but also in vitro in experiments with mammalian reticulocytes, duck

erythrocytes and with hemolysates (London and Yamasaki, 1952). It appears, therefore, that some of the glycine disappearing from the blood and liver of the intoxicated chick, as well as part of the introduced glycine, might be diverted to the biosynthesis of replacement hemoglobin.

The marked reduction in the hemoglobin concentration of chicks intoxicated with pullorum endotoxin or infected with S. pullorum during the first twelve hours (Figure 7) could represent a counter-shock response of the host. The early stages of intoxication are characterized by profound vascular disturbances (Boquet et al., 1947). These disturbances are marked by waves of arteriolar constrictions and dilations. Since vasodilations alone have been shown to result in hemadilution as a consequence of increased plasma volume (Walsh et al., 1956), this circulatory change might be responsible for the apparent reduction in the hemoglobin concentration observed in intoxicated and infected chicks during the early phase of the experimental period.

It is of interest to note at this point that the bleeding of chicks intoxicated with pullorum endotoxin or infected with S. pullorum became more and more difficult as time passed. This could be indicative of vascular disturbances and abnormalities in the blood coagulation processes. This point awaits further investigation. Takeda et al. (1955) noted similar difficulties with rabbits intoxicated with the endotoxins of S. typhosa, S. paratyphi B and Shigella flexneri. The administration of adensosine triphosphate (ATP) removed the difficulty.

It is known that disturbances in circulation result in functional changes in the liver. In both clinical and experimental shock there is evidence of liver damage. In experimental hemorrhagic shock there is a sharp decrease in portal blood resulting in anoxia of the liver and disturbance of amino acid metabolism. Likewise, in chronic passive congestion, stasis and anoxia may induce functional and anatomical changes in the liver. Also, interference with the hepatic circulation can lead to massive necrosis. The mechanism of toxic hepatic necrosis has been widely investigated. One theory, recently supported by Cameron (1953), attributes the effect of hepatotoxic agents such as carbon tetrachloride and fluoroacetate to direct interference with cellular metabolism. Another theory, supported mainly by Himsworth (1947), attributes centrilobal necrosis to local anemia of the cells surrounding the central vein of the liver. Carbon tetrachloride necrosis according to this theory, is the result of reduced blood supply due to restriction of sinsoids by swollen parenchymal cells. Stoner (1956) studied the mechanism of hepatic necrosis, produced by a number of toxic agents, through the application of internal calorimetry. Carbon tetrachloride, beryllium sulfate, allyl formate, 3:5 dinitro-o-cresol and fluoroacetate were studied as hepatotoxic agents in rats. The results of the investigation were considered to be additional evidence in support of the theory that the lesions of toxic hepatic necrosis are the direct result of interference with the metabolism of liver cells.

Although fluoroacetate is best known as an inhibitor of the

tricarboxylic acid cycle (Peters et al., 1953), the compound in sub-lethal doses will produce hepatic necrosis (Hicks, 1950). This fact is of interest since hepatic necrosis marked by fatty infiltration has been shown to be characteristic of the pathology of pullorum disease in chicks (Jones, 1909). Evidence of fatty infiltration and focal necrosis of the liver was obtained in the course of this investigation of the effect of endotoxin on the physiology and metabolism of the chick.

Since uncoupling of oxidative phosphorylation has been reported in fatty livers (Dianzani, 1954), it seemed necessary to consider this as a possible mode of action of endotoxin in producing biochemical alterations within the host. It has been shown that many of the substances which inhibit oxidative phosphorylation also produce fatty infiltration of the liver when injected into experimental animals (Dianzani and Scuro, 1956). A decrease in adenosine triphosphate (ATP) and of phosphocreatine has been shown to occur in many of the organs of rats treated with 3:5 dinitro-o-cresol, a substance which inhibits oxidative phosphorylation and is a hepatotoxin (Parker, 1954). From these observations one might expect to find a decreased concentration of ATP in fatty livers.

The results of this investigation support, indirectly, the idea that the relationship between the occurrence of fatty liver and the inhibition of the adenylic system is more than casual in chicks intoxicated with pullorum endotoxin. There is evidence indicating that endotoxin blocks the normal activity of the tricarboxylic acid

and consequently reduces the available supply of ATP. The increase in the inorganic phosphorus in the blood of the intoxicated chick (Table 10) could be indicative of the breakdown of high energy phosphorus containing compounds. Takeda et al. (1955) observed that the increase in inorganic phosphorus in the blood of intoxicated rabbits was accompanied by a decrease in organic phosphorus. All of these changes might be interpreted to indicate that endotoxin alters phosphorus metabolism. The inhibition of the regeneration of ATP due to the energy deficiency caused by the blocking of the tricarboxylic acid cycle is strongly suggested by all of these observations.

Additional evidence supporting the idea of the inhibition of the adenylic system by endotoxin was recently obtained by Takeda et al. (1955). These investigators found that the lethal action of highly purified endotoxins derived from several different gram-negative organisms could be counteracted by the injection of appropriate doses of adenosine triphosphate (ATP). Approximately the same dose of ATP was required to prevent the death of rabbits regardless of the source of the endotoxin. The injection of ATP also reversed most of the abnormal metabolic changes in animals normally produced by endotoxin including increases in lactic acid in the blood, increases in inorganic and decreases in organic phosphorus in the blood, and decreases in alpha-ketoglutarate of the blood. Hyperglycemia, however, could not be prevented by the injection of ATP. This might be due to the fact that both endotoxin and ATP cause a temporary acceleration of the oxidative metabolic processes and therefore cause the reserve glycogen to be mobilized and utilized rapidly.

Inhibition of the adenylic system could also serve to help explain the fatty infiltration of the livers of intoxicated and infected chicks. Since ATP is essential for the activation of fatty acid oxidation, it is probable that the fatty infiltration is a direct result of inhibition of the adenylic system. Under the conditions of intoxication and infection the oxidation of fats could not occur. The result would be an accumulation of fats which were mobilized and moved to the liver under the influence of endotoxin. Dianzani (1957) observed that a decrease in ATP preceded the development of fatty livers in experimental animals.

Two other conditions might also be responsible for the development of fatty livers in intoxicated and infected chicks. A decrease in the transport of phospholipids from the liver, due to a deficiency of lipotropic factors such as methionine and choline, has been shown to contribute to the development of fatty livers in experimental animals. Also, the increased migration of fat from the depots of the liver, in the absence of oxidation, has been shown to contribute to fatty infiltration of the liver (Stetten and Salcedo, 1944). A decrease of methionine from the blood and liver of intoxicated and infected chicks (Figures 3, 4, and 5) could represent a reduction of lipotropic factors available to the chick and could result in an impairment of the movement of phospholipids from the liver.

Lipid deposits have been found to occur in the livers of experimental animals during the alarm phase of stress induced by a number of agents including bacterial toxins. Total blood lipids

have been shown to decrease during stress and it appeared that fat was being transferred from depots to the liver (Foglia and Selye, 1938; Leblond et al., 1939).

It appears, therefore, that the fatty liver developing in chicks injected with the endotoxin of S. pullorum and infected with S. pullorum might be attributable to the combined action of several factors. Included are the following: (1) increased mobilization of depot fats; (2) a reduction in the transport of fats from the liver due to a decrease in lipotropic factors; and (3) impaired utilization of fats in the liver due to the reduction in the regeneration of ATP.

The results of this investigation show that cortisone has the property of disorganizing the natural host-parasite relationship with the outcome being shifted overwhelmingly to favor the parasite. The survival time of infected chicks was reduced by the injection of this hormone (Table 13) while the survival time of the intoxicated animals was extended by similar treatment. The infectious capacity of several representative species of bacteria has been shown to be enhanced by cortisone treatment of the host (Thomas, 1952, 1953) and this observation has now been extended to specifically include S. pullorum infection in baby chicks.

The significance of the protective action of cortisone against the lethal effects of endotoxin, under the conditions of these experiments, is difficult to analyze. Only further investigation can determine whether cortisone influences endotoxic activity in established natural infections. Also, the extent to which cortisone

alters the relationship between the adverse effects of the corticosteroids on the mechanisms of resistance to infection on one hand, and the protective actions of these steroids against the lethal action of endotoxin on the other hand, remains to be determined.

In connection with the protective effect of cortisone against the lethal action of endotoxin, it is interesting to note that this hormone has been found to increase the activity of the enzyme arginase (Fraenkel-Conrat et al., 1943; Kochakian, 1945, 1951). Also, the administration of cortisone has been shown to increase the rates of formation and excretion of creatinine, urea, and uric acid (Sprague et al., 1950; Ingbar et al., 1951; Bishop et al., 1951; Engle, 1953). Thus, it would appear that cortisone could exert its protective effect against endotoxin by accelerating the ornithine cycle in the liver of experimentally treated chicks. Cortisone has also been shown to protect animals against experimentally induced fatty liver (Kupperman et al., 1955).

The results of this investigation, although preliminary in nature, indicate that the endotoxin of S. pullorum plays an important role in the metabolic and physiological alterations accompanying this infection in baby chicks. The study also serves to emphasize the possible role of endotoxin as the biochemical determinate of disease, especially in the case of gram-negative bacterial infections. Deaths in many such infections are often attributed to irreversible changes in the tissue which have occurred prior to the administration of effective antibiotic therapy. The unsatisfactory nature of this

explanation has made it necessary to attempt to characterize the underlying biochemical and physiological disturbances induced in the host by microorganisms or their toxins. Once the nature of these disturbances is understood, treatment on a more fundamental basis can be instituted. Following this line of reasoning, one might conceive of the future treatment of serious gram-negative infections as involving the use of antibiotics to control bacterial growth and the administration of essential metabolites and substrates shown by investigation to be reduced in concentration by the infectious process. The replacement of these metabolites or substrates would permit the proper functioning of the cells of the host during the critical phase of infection. Through the use of this type of treatment it might be possible to eliminate the "therapeutic paradox" of a host dying while being "bacteriologically cured."

CHAPTER VI

SUMMARY

The role of endotoxin in the pathology of a gram-negative bacterial infection has been studied in a model host-parasite system involving the baby chick and *S. pullorum.*

Alterations in the amino acid metabolism, nitrogen excretion patterns, and the concentrations of selected reactants have been studied in chicks intoxicated with the specific endotoxin of *S. pullorum.* These alterations have been correlated with the metabolic and physiological changes in the chick which are characteristic of pullorum disease.

The concentrations of the essential amino acids arginine, glycine, and methionine have been found to be reduced in the blood and liver of intoxicated chicks during the experimental period. These reductions in the concentrations of the amino acids were accompanied by increases in the concentrations of urea, uric acid, creatine, and ammonia in the blood of intoxicated chicks. Blood sugar and the serum inorganic phosphorus increased during experimental intoxication, while the hemoglobin concentration was found to be decreased.

The administration of glycine to intoxicated chicks resulted in an extension of survival time and prevented, at least in part, the reduction in hemoglobin concentration in latter stages of the experimental period.

The administration of arginine to intoxicated chicks resulted

in an extension of survival times and produced corresponding increases in the concentrations of blood urea. The increased blood urea concentrations were marked by a concomitant reduction in the levels of ammonia in the peripheral blood of chicks intoxicated with the endotoxin of S. pullorum.

On the basis of the results of this investigation, a hepatic system has been proposed which accounts for the disappearance of arginine, glycine, and methionine. The hepatic system, as proposed, included the reactivation of the Krebs "ornithine cycle" in the liver of the experimental chick through the action of endotoxin. The participation of arginine in this cycle and in the biosynthesis of creatine, appeared to permit the removal of ammonia from the blood by converting it into urea and creatine. The protective effect of arginine appeared to be exerted in this manner. In addition, blood ammonia appeared to be converted into uric acid in the livers of chicks intoxicated with endotoxin at an increased rate.

The concentrations of citrate were measured in the livers of normal and intoxicated chicks. Citrate appeared to accumulate in the livers of intoxicated chicks and this alteration was accepted as evidence of a block in the tricarboxylic acid cycle attributable to the action of the endotoxin.

The concentration of coenzyme A was found to be reduced by the injection of endotoxin into chicks. It appeared, on the basis of these results, that the effective entry of pyruvate into the tricarboxylic acid cycle was prevented through the action of endotoxin.

The results of this investigation on the mode of action and the metabolic changes produced by endotoxin suggested disturbances in the phosphorus metabolism of the chick. Also, the disturbances in the metabolic processes of the chick suggested that oxidative phosphorylation was inhibited by endotoxin. It appeared that the regeneration of high energy phosphorus compounds was inhibited by the action of endotoxin, which in turn resulted in the inhibition of phosphorylation and other energy requiring reactions in the oxidative assimilation of carbohydrates and other metabolites.

The possible role of endotoxin as a biochemical determinate of gram-negative bacterial diseases is discussed.

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