8-1964

The effects of amino acid supplementation and medication of salmonella pullorum in chicks

Ronald D. Simpson

To the Graduate Council:

I am submitting herewith a thesis written by Ronald D. Simpson entitled "The effects of amino acid supplementation and medication of salmonella pullorum in chicks." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Animal Husbandry.

R.L. Tugwell, Major Professor

We have read this thesis and recommend its acceptance:

O.E. Goff, J.N. Liles

Accepted for the Council:
Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

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

July 31, 1964

To the Graduate Council:

I am submitting herewith a thesis written by Ronald Dale Simpson entitled "The Effects of Amino Acid Supplementation and Medication on Salmonella Pullorum in Chicks". I recommend that it be accepted for nine quarter hours of credit in partial fulfillment of the requirements for the degree of Master of Science, with a major in Poultry.

R. L. Ingwell
Major Professor

We have read this thesis
and recommend its acceptance:

O E Goff

James M. Giles

Accepted for the Council:

Clifton A. Smith
Dean of the Graduate School

THE EFFECTS OF AMINO ACID SUPPLEMENTATION AND MEDICATION ON *SALMONELLA PULLORUM* IN CHICKS

A Thesis

Presented to

the Graduate Council of

The University of Tennessee

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

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by

Ronald D. Simpson

August 1964

ACKNOWLEDGEMENT

The author wishes to express his appreciation to the members of his graduate committee, Drs. R. L. Tugwell, O. E. Goff and J. N. Liles. To Dr. Tugwell, whose guidance and assistance has contributed much to this research, particular appreciation is acknowledged.

The author wishes to further express his gratitude to Dr. D. F. Holtman and Dr. G. E. Hunt for their extremely valuable suggestions and technical assistance which formed a basis for much of this work.

In addition, the writer is grateful for the unselfish help of his colleagues, Mr. C. R. Douglas and Mr. R. A. Voitle.

Finally, the author wishes to express his deep appreciation to his wife, Charlotte, whose encouragement and constant support helped make this study possible.

TABLE OF CONTENTS

PAGE

PAGE

LIST OF TABLES

LIST OF FIGURES

FIGURE PAGE

INTRODUCTION

The widespread and sometimes indiscriminate use of drugs and medicants has, in part, led to masking of the physiological and biochemical mechanisms involved in host-parasite relationships in certain bacterial diseases. Additional knowledge is needed in relation to physiological alterations with emphasis on the metabolic disturbances within the host during infection.

The literature suggests that certain infectious diseases can cause alterations in protein, carbohydrate and fat metabolism. 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.

Although pullorum disease has been largely controlled by testing programs, there still exists a potential danger to the poultry industry from this disease. There is still no known treatment for pullorum disease in poultry that can guarantee complete recovery and control. Consequently, more needs to be known regarding host-parasite relationships, drug action and therapeutic evaluation of drugs in its control. Furthermore, this knowledge might well be applied to other bacterial infections.

Dubos (1955) has done much to renew the interest in the biochemical basis to infection. With the metabolic disturbances in mind, Ross *et al.* (1955a) and Dooley *et al.* (1956) reported

some major alterations in the nitrogen metabolism of chicks clinically infected with Salmonella pullorum. Among this findings was the observation that certain free and bound amino acids in the blood serum were depleted during the course of infection. Subsequent supplementation of some of the depleted amino acids resulted in prolonging the survival time of the fatally infected chicks.

Much work has been done with dietary protein levels and disease interrelationships. However, little has been reported with respect to individual amino acids and disease interaction. The study reported in this thesis was undertaken to explore the aspects of amino acid deficiencies; supplementation of serum-deficient amino acids; and drug evaluation in search for a combined treatment that might be beneficial in treating chickens with pullorum disease.

REVIEW OF LITERATURE

Pullorum Disease

Pullorum disease was first recognized as a bacterial infection by Rettger (1900). He described the disease as a fatal septicemia of young chicks. In a subsequent report Rettger and Stoneburn (1909) applied the term "Bacillary White Diarrhea" and proposed the etiological agent be designated Bacterium pullorum. In 1918 the Society of American Bacteriologists changed the name of the microorganism from Bacterium pullorum to Salmonella pullorum. In 1929 the name of the infection was changed to pullorum disease for brevity and specificity.

At the close of the nineteenth century the disease was a serious menace to the poultry industry and resulted in major economic losses. In 1935 the United States Department of Agriculture accepted the responsibility of administering the National Poultry Improvement Plan for the purpose of control and eradication of pullorum disease. The economic importance of this disease has encouraged considerable investigation regarding its diagnosis and epidemiology. Rettger and Stoneburn (1909) established the fact that S. pullorum is an egg borne organism and is involved in cyclic transmission. Weldon and Waver (1930) showed that the infection could be spread from chick to chick during early cohabitation.

Runnels and Van Roekel (1927) reported that 33.7 percent of the eggs laid by reactor hens were infective. Bushnell et al. (1926), Dearstyre et al. (1929), Runnels (1929) and Kerr (1930) reported that pullorum disease in adult birds caused a reduction in fertility, hatchability and egg production and that apparently healthy hens could be chronic carriers of the organisms. Furthermore, these carriers seemed to be less able to withstand changes in environmental conditions and concurrent flock diseases. Attempts to raise chicks from infected adult stock were difficult and disappointing.

The symptoms and lesions of pullorum disease are well known as reported by Van Roekel (1959). Much less is known concerning the biochemical alterations during the course of infection with reference to host-parasite interactions, metabolic disturbances, amino acid imbalances and drug action during treatment.

Protein Metabolism

The subsequent necrotic lesions of pullorum disease are suggestive of possible alterations in one or more metabolic processes. Almquist (1947) divided the amino acid requirements of the chick into three categories: (1) indispensable, (2) dispensable and (3) those required in certain cases for their sparing effect on certain of the indispensable amino acids. The essential and indispensable nature of certain amino

acids in the metabolism of the chick prompted investigations concerning their alterations during an infectious disease.

Berry and Mitchell (1953) observed that alterations in the tricarboxylic acid cycle significantly altered the survival time of mice infected with Salmonella typhimurium. Woodward et al. (1954) observed that rats infected with Pasteurella tularensis exhibited a reduction in all free amino acids in the serum when analyzed by two-dimensional paper chromatography. Likewise, there was a consistent failure to detect cystine, arginine, and phenylalanine. Ross et al. (1955a) reported that one to three day-old chicks infected with S. pullorum showed a reduction in arginine, glycine, methionine and tryptophan in both the free and bound amino acids in the blood serum when analyzed by two-dimensional paper chromatography. There was a consistent detection of 16 amino acids in the bound protein and 13 amino acids in the free amino acids of the serum.

Dooley et al. (1956) also reported that endotoxin extracted from S. pullorum and injected into day-old chicks resulted in a reduction of glycine, methionine and arginine in the free and bound serum amino acids of these intoxicated chicks. Paralleling these reductions was an increase in the levels of non-protein nitrogen, urea, creatinine, uric acid, ammonia and inorganic phosphorus.

Ross et al. (1955b) reported that supplementation of arginine both orally and intreperitoneally resulted in a pronounced increase in survival time of the inoculated chicks

infected with pullorum. A slight increase in survival time was noted in the group given supplementation of methionine and the group receiving a combination of arginine, methionine, glycine and tryptophan. No significant increase in survival time resulted in glycine or tryptophan supplemented chicks. Gilfillan (1955) observed that none of these four amino acids was required by the microorganism. He stated that the parasite would utilize them but "preferred" other amino acids when a choice was possible. These findings suggest that the host might utilize the amino acids as a defense mechanism against the infection rather than the parasite depleting them *per se* for its own needs as was first thought.

Role of Arginine, Glycine, Methionine and Tryptophan

Much is known, as Cannon (1950) reports, regarding the indispensable and necessary nature of the amino acids in the production of plasma globulins which in turn are used in the synthesis of antibodies. Far less is known regarding the individual role and mechanism of the serum-deficient amino acids in metabolism during infection.

Patch (1936) noted that the addition of arginine, along with cystine, to a casein-milk powder diet caused a striking and prolonged increase in erythrocytes and hemoglobin production. Arnold *et al.* (1936) demonstrated that arginine has a profound effect on the metabolism of growing chicks. Klose and Almquist (1940) reported that citrulline is able to replace supplemental

arginine while ornithine cannot be converted to arginine by the chick. Hegsted et al. (1941) indicated that arginine is required for creatine formation. Almquist (1947) established that the optimum level of arginine in the normal diet is 1.2 percent. The precursory role of arginine in the synthesis of uric acid and creatinine was confirmed by Ross (1956) when he observed significant increases in uric acid and creatinine within 12 hours after infection while further observing a paralleled decrease in arginine for the same period. Of even greater significance and importance is the fact that greater increases in blood urea of infected birds occurred in arginine treated birds.

Further investigations by Ross (1956) indicate that the enzyme arginase is operative during infection in catalyzing the synthesis of urea from arginine via the reactivated ornithine-citrulline cycle in the liver, also activated during infection. This may explain the increase in blood urea in infected birds and perhaps add support to the postulation that the urea cycle is activated during infection. While arginine is neither toxic to the host or parasite its therapeutic value could well lie in the fact that the formation of urea can inhibit the growth of S. pullorum and eventually become toxic to both parasite and host. Dooley (1957) further adds to the protective role of arginine by reporting its ability to remove excess and potentially toxic ammonia from the peripheral blood

of infected chicks by converting it to urea and creatine in the hepatic system.

Glycine is known to participate with arginine in the formation of uric acid and creatine. Sonne et al. (1946) stated that glycine may act as the donor of carbons 5 and 6 in uric acid. Ross (1956) reports that glycine serves as a precursor in the synthesis of hemoglobin and that this may be very significant in the overall host response. Dooley (1957) observed that the administration of glycine to the intoxicated chicks appeared to prevent, at least in part, the marked reduction in hemoglobin between 24 and 60 hours post inoculation. It therefore appears that some of the glycine disappearing from the blood and liver during intoxication might be diverted to the biosynthesis of replacement hemoglobin.

Norris and Scott (1952) cited that methionine served as a source of methyl groups in carrying out the methylation process and either exerted a sparing effect on choline or took part in the synthesis of choline. As methionine is a known precursor of choline and as choline deficient diets exhibit the fatty liver syndrome it is conceivable that alterations in concentrations of methionine might result in fatty liver degeneration during pullorum infection.

Briggs et al. (1946) found that tryptophan could replace nicotinic acid and overcome the growth depressing effects of a low nicotinic acid diet. Schweigert et al. (1948) provided evidence that tryptophan was converted into nicotinic acid

in the developing embryo.

Medication of Pullorum Disease

Beach and Freeborn (1927) recorded that drugs and chemicals including chinosol, metaphen, sulfuric acid, hydrochloric acid, mecuric chloride, resorcin, potassium permanganate, sulfocarbolates, and hypochlorite solutions had no beneficial influence in combatting pullorum disease when taken into the alimentary tract. Work of Grumbles et al. (1940); Severens et al. (1945); Bottorff and Kiser (1946); Anderson et al. (1948); Cole (1948); Dickinson and Stoddard (1949); and Cooper et al. (1951) indicate that the introduction of sulfanomides in the control of the infection may be effective in reducing mortality. However, these drugs failed to prevent a retardation in the growth associated with the disease. Likewise, the incidence of reactors among survivors was not reduced by administration of these drugs.

The value of antibiotics in the control of pullorum disease is questionable and vague. Biotherapy (vaccines, bacterins and serums) have not given satisfactory results. The extensive use of formaldehyde as an incubator fumigant is known to be quite effective in control of the disease during incubation.

Recently NF-180[1] has been found to have considerable

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[1]Trade name of furazolidone, a product of Hess and Clark Company.

merit in preventing losses from the disease. Recent investigations of Smith (1954), Gordon and Tucker (1955), Wilson (1955, 1956), Smyser and Van Roekel (1957) and Bierer (1961) have borne this out. However, work by Tugwell *et al.* (1959) showed that viable *S. pullorum* organisms were recovered five and a half months after infection and treatment with NF-180 in Single Comb White Leghorn pullets.

More recently, claims have been attached to a new sulfonamide called SEZ[2] in the treatment of *Salmonella gallinarium* and *Salmonella typhimirium*. The value of SEZ as a treatment in pullorum disease has not been reported.

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[2]Trade name of sulfaethoxypyridesine, manufactured by American Cyanamid Company.

MATERIALS AND METHODS

Experiment I

The Effect of Pullorum Disease on the Free Amino Acids in Blood Serum

Standardization of Inocula

A Salmonella pullorum strain (CDC 3763-62) was obtained from the Communicable Disease Center, Atlanta, Georgia. The strain was maintained by the freeze-dry process.

Nutrient agar slants were streaked with a broth culture of the microorganisms and incubated at 37° C. for 24 hours. The slants were then washed with sterile physiological saline and the bacteria harvested in the saline suspension. Graded turbidity readings on the Klett-Summerson colorimeter were taken of the suspended bacteria and plated on Salmonella Shigella agar plates. After incubating 48 hours at 37° C. the plates were examined and individual colonies counted with the aid of a Quebec Counter. Plate counts were then correlated to the turbidity readings of the colorimeter. In preparing the inocula, bacteria were incubated for twenty-four hours on nutrient agar slants and harvested by washing with a physiological saline solution then adjusted by dilution to a given Klett-Summerson reading.

Initial Infection with S. pullorum

A total of 200 Single Comb White Leghorn female chicks,

University of Tennessee strain, were used in Experiment I. The chicks were vaccinated at one day of age for Newcastle disease and infectious bronchitis by the intraocular method and held in heated battery brooders at the Poultry Service Laboratory until two weeks of age. All chicks were fed a 22 percent protein basal diet void of all drugs and feed additives.

An LD_{50} dosage of S. pullorum was calculated and administered intraperitoneally to 150 of the chicks at two weeks of age. The inoculum consisted of 10^5 cells suspended in 1.0 ml. of physiological saline solution. Fifty chicks received no inoculation and were maintained as controls.

Collection of Blood Samples for Chromatographic Analysis

Blood samples were taken from the survivors of the infected birds at 24, 48, 72 and 96 hours post inoculation. At the same time samples were taken from the noninfected controls. During each collection period 3 ml. of blood were taken from each of twenty-four inoculated chicks and nine noninoculated chicks by frontal heart puncture. Three initial blood samples were then pooled into one composite sample for chromatographic analysis. The blood tubes were slanted, allowed to clot for three to four hours at room temperature, placed at 3° C. overnight and finally the serum separated into clean tubes and stored in a freezing compartment at -6° C. until used for chromatographic spotting samples.

Chromatographic Procedures for the Determination of Free Amino Acids in the Serum

Equal portions of blood sera and acetone were mixed and the protein allowed to precipitate. The samples were then centrifuged for one minute and 0.1 ml. of the supernatant removed by pipette for the spotting sample (Popp 1963). Whatman number one filter paper was used as chromatographic paper. The spotting sample was applied to the corner of the 18 inches by 22 inches sheet approximately four inches from either margin. The sample was immediately allowed to dry. Warm circulating air from a small electric heater was used to aid drying.

Two-dimensional paper chromatography was used, employing the descending technique (Block et al. 1956). The first solvent used was 80 percent phenol in water. The sheets were dried at room temperature by circulating air and turned for the second run using butanol-acetic acid-water in a 4:1:1 ratio. Again the sheets were dried and the amino acid spots developed by 0.25 percent ninhydrin in acetone. RF[3] values of the amino acid spots were determined and compared to the RF values of known standards.[4] The intensity and size of the spots from samples of the infected group were compared to those of the noninfected control group. Alterations in amino

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[3]Relative factor, used in comparing distance of movement of the individual amino acids on chromatographic paper.

[4]Obtained from Dr. Gordon E. Hunt, University of Tennessee, Botany Department.

acid concentrations were determined by this comparison.

Experiment II

The Effects of Supplementation of the Serum-Deficient Amino Acids to Infected and Noninfected Chicks

A total of 432 Single Comb White Leghorn female chicks were used in this experiment. They were obtained and maintained in the same manner as the previous experiment. Based on the findings of the previous experiment, 12 groups of 36 two-week old chicks were established. Each group was placed in battery brooders in triplicate, according to a randomized block design. Six groups were inoculated with S. pullorum and were paired with six control groups receiving no inoculation. Both the inoculated and noninoculated groups were placed on treatments of supplemental (1) arginine, (2) glycine, (3) methionine, (4) tryptophan, (5) a combination of arginine, glycine, methionine and tryptophan, and (6) no supplementation. The birds were inoculated intraperitoneally with a standardized inocula, calculated at an LD_{50} dose rate. Inoculation occurred at two weeks of age and the experiment was terminated at fourteen days post-inoculation.

The hydrochloride forms of the amino acids were used. Oral supplementation was employed with each bird receiving 200 mg., dissolved in two ml. of sterile distilled water, of the designated amino acid each day for four successive days

following inoculation. In the case of the combined amino acid supplementation, 50 mg. of each amino acid was mixed, totaling 200 mg. per bird per day. In each instance the amino acid solutions were placed in the crop of the inoculated chicks with the aid of a 2 ml. pipette.

Chromatographic Analysis

Blood samples were taken at 24, 48 and 72 hours post inoculation. Three birds from each replicate were designated as blood donors. One ml. from each bird was pooled into one composite sample for each replicate at each of the three collection periods. Blood sera were separated and frozen for future chromatographic analysis. An attempt was made to study the level or amount of reduction of each amino acid as influenced by the course of the infection and individual supplementation. The same method of two-dimensional paper chromatography was employed as previously described.

Mortality Percent

Mortality was recorded for each group. Total number of deaths and average hour of death was calculated for each group. These data were used as comparisons between various group supplementations.

Morbidity Percent

Morbidity percent was observed and recorded during the course of the infection. All symptoms, gross pathology, behavior

and relative speed of recovery were recorded and compared between groups.

Feed Consumption

Feed consumption per pen per week was recorded and the average consumption per bird was calculated for each of the noninoculated groups. Complete or partial mortality in some of the inoculated replicates rendered calculation difficult or impossible in these groups.

Average Weight Gains

Individual chick weights were taken just prior to inoculation and again at the end of the experiment, two weeks post inoculation. The weight gain in grams was calculated for each bird for the two week period. Average weight gain per bird per replicate was calculated and used as a criterion for comparison between groups.

Experiment III

The Comparative Effects of Selected Amino Acid Supplementation and Medication on *S. pullorum* Infection in Chicks

A total of 585 two-week old female chicks were used. The University of Tennessee strain of Single Comb White Leghorns was used. The chicks were vaccinated at one day of age for Newcastle disease and infectious bronchitis by the intraocular method and held in heated battery brooders until two weeks of age.

At two weeks of age the chicks were randomized, wing banded and assigned to the various treatments after being inoculated with 1.0 ml. of a LD_{50} dosage of S. pullorum. One treatment, maintained as a control, received no inoculation. After inoculation at two weeks of age the birds were maintained until six weeks of age, at which time the trial was terminated.

NF-180, SEZ, arginine and methionine were used in various combinations with a 22 percent protein basal all mash diet containing no drug additives. In this trial arginine and methionine were mixed in the feed in combination with SEZ or NF-180. There was a total of 13 treatments in this trial. Each treatment consisted of 45 chicks which were divided into three replicates of 15 chicks each. Table I shows the 13 treatments. Treatment replicates were arranged in a complete randomized design.

The noninfected controls were kept in a separate room maintained at the same temperature and under as nearly the same conditions as were the infected birds. Precautions were taken to prevent contamination or disease transmission from infected birds to the noninfected control birds.

NF-180 was administered at 200 grams per ton of feed for the first four days post inoculation. On the fifth day this level was decreased to 100 grams per ton of feed and held at this level for 10 additional days. At 14 days post inoculation medication ceased and the birds were maintained on the basal

TABLE I

AMINO ACID SUPPLEMENTATION AND MEDICATION, EXPERIMENT III

Treatment No.	Treatment	Infection status
Group 1		
1	Basal	Infected
2	Basal + arginine	Infected
3	Basal + methionine	Infected
4	Basal + arginine + methionine	Infected
Group 2		
5	Basal + NF-180	Infected
6	Basal + NF-180 + arginine	Infected
7	Basal + NF-180 + methionine	Infected
8	Basal + NF-180 + arginine + methionine	Infected
Group 3		
9	Basal + SEZ	Infected
10	Basal + SEZ + arginine	Infected
11	Basal + SEZ + methionine	Infected
12	Basal + SEZ + arginine + methionine	Infected
Group 4		
13	Basal (control)	Noninfected

diet for the latter two weeks of the trial.

SEZ was administered at 500 grams per ton of feed for four days post inoculation. On the fifth day this level was reduced to 250 grams per ton of feed and held for 10 additional days. At 14 days after the initial inoculation medication ceased and the birds were maintained on the basal diet for the duration of the trial.

Arginine and methionine were supplemented by addition to the all mash diet. Three grams of the pure amino acid were added to each pound of feed. In the case of the combined amino acid treatment 1.5 grams of each were added to the feed, totaling three grams per pound.

Data were subjected to statistical analysis using analysis of variance (Snedecor 1956). Duncan's New Multiple Range Test (Steel and Torrie 1960) was used in the comparing of several treatment means. The following criteria were used as comparison between groups.

Mortality Percent

Mortality was recorded at 12 hour intervals throughout the four-week trial. Mortality was then calculated by percent for each replicate. Percent mortality of each treatment represented a criterion for comparison of treatment effects on pullorum in chicks.

Morbidity Percent

Morbidity observations were recorded each 12 hours after

infection occurred until all birds had apparently recovered. The peak of morbidity for each group and relative time of apparent recovery for each group was recorded. Visual symptoms and behavior of all chicks were noted. Morbidity was expressed in percent for each treatment and thus served as a criterion for comparison.

Average Weight Gains

All chicks were weighed at two weeks of age just prior to inoculation and at four and six weeks of age. The weight gain in grams was calculated for each bird from two weeks of age to four weeks of age and again from four weeks of age to six weeks of age. The average weight gain for birds in each treatment was then calculated and used as a criterion for comparison between groups.

Average Feed Consumption

Feed consumption records were maintained from time of inoculation until the termination of the trial for each replicate. These data were converted into the average number of grams consumed per chick for each two week period. The average number of grams consumed per chick for the entire four week period was also calculated for each treatment and used as a criterion for comparison of all treatments.

Blood Titers

At four weeks post inoculation antibody blood titers were taken from 20 surviving birds of each group. The rapid whole

blood plate test was used with a polyvalent K stained pullorum-typhoid antigen. The arbitrary values representing the strength of reaction were recorded from 0 through 4. Zero represented a negative reaction, one represented a slight agglutination within 90 seconds, two represented a somewhat stronger reaction, while four represented an immediate agglutination with maximum clumping. The blood titers were averaged for each group and used in evaluating antibody production for each treatment.

Recovery of Viable Organisms

Following the determinations of antibody blood titers the same birds were then sacrificed and an attempt made to recover viable S. pullorum organisms. Culturing was accomplished by using a flamed bacteriological loop or needle and sterile tubes of nutrient broth. Tissue cultures were taken from the (1) heart, (2) liver, (3) spleen, and (4) bone marrow of each chick by stabbing the sterile loop or needle into the tissue and aseptically transferring to the tubes of nutrient broth. A tube of medium was provided for each individual culture. The nutrient broth cultures were then incubated at 37° C. for 24 hours. Samples from all positive tubes were then streaked on S. S. agar plates and incubated for 48 hours at 37° C.

Salmonella colonies were identified by observation and with the use of the Quebec Counter. The colonies were recorded and finally transferred to differential media; such as SIM, TSI, urease and a series of sugars, in an attempt to positively identify the microorganism as S. pullorum. Identification in

these cases were based on sugar fermentation, acid formation, gas production, H_2S production, motility and decomposition of urea.

A total of 1040 individual cultures was taken from 260 different birds. These data were used as criterion in the evaluation of the various treatments.

RESULTS AND DISCUSSION

Experiment I

The Effect of Pullorum Disease on the Free Amino Acids in Blood Serum

Standardization of Inocula

Numbers of organisms observed by the plate count method were correlated with the turbidity readings of the suspensions from which dilutions were made. A reading of 105 on the Klett-Summerson colorimeter when diluted to 1-100,000 corresponded with 10^5 organisms per ml. of suspension. One ml. of this inoculum per chick via the intraperitioneal route served as the LD_{50} number of cells when calculated for two-week-old Single Comb White Leghorn females.

Chromatographic Analysis of the Free Amino Acids in the Blood Serum

Chromatographic analysis of the free amino acids in the blood of infected chicks revealed a decrease in the concentration of certain amino acids. A total of 12 amino acids appeared consistently in the serum of the control chicks (Figure 1). Traces of two other amino acids, tryptophan and serine, appeared irregularly. Chromatograms from the blood of infected chicks when compared with chromatograms from control chicks showed a defininte reduction in the concentration of arginine,

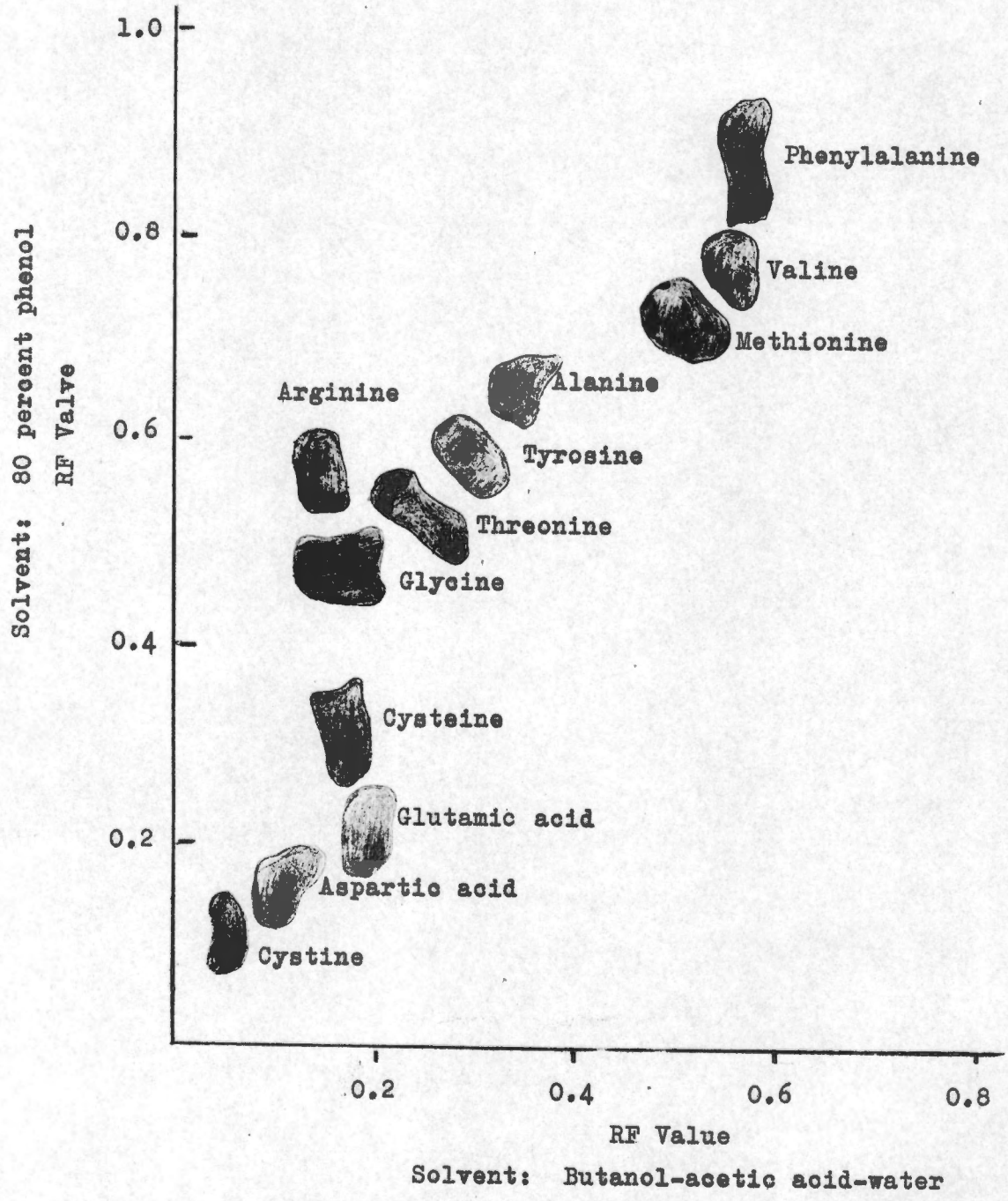

Figure 1. Free Amino Acids in Blood Serum of Noninfected chicks.

methionine and glycine (Figure 2). This was evident by the marked decrease in color intensity and size of the spot when developed with ninhydrin. By further dilution of the supernant employed as the spotting sample, it was possible to nearly eliminate these three amino acids from the chromatograms, while the other nine remained relatively intense in color.

All three amino acids appeared to be more greatly reduced 48 and 72 hours after inoculation than at 24 and 96 hours. These data confirm the findings of Ross et al. (1955a), with the exception of tryptophan. However, Ross (1955a) used one to three day-old chicks and administered a lethal dose of S. pullorum to all chicks.

The blood serum level of arginine appeared to be moderately reduced at 24 hours post inoculation with the greatest reduction occurring between 48 and 72 hours. At 96 hours a pronounced but not complete recovery was apparent. Free methionine in the blood serum also appeared moderately reduced at 24 hours. Methionine exhibited a greater reduction at 48 hours than did arginine, but revealed at 72 hours a greater ability to partially recover than did arginine. At 96 hours the methionine level was shown to be almost completely recovered; more so than arginine at this time. Alterations in glycine appeared more gradual and less intense when compared to arginine and methionine. Glycine reduction at 24 hours was very slight and sometimes questionable. At 48 hours post inoculation glycine showed only moderate reduction and at 72 hours again only slight

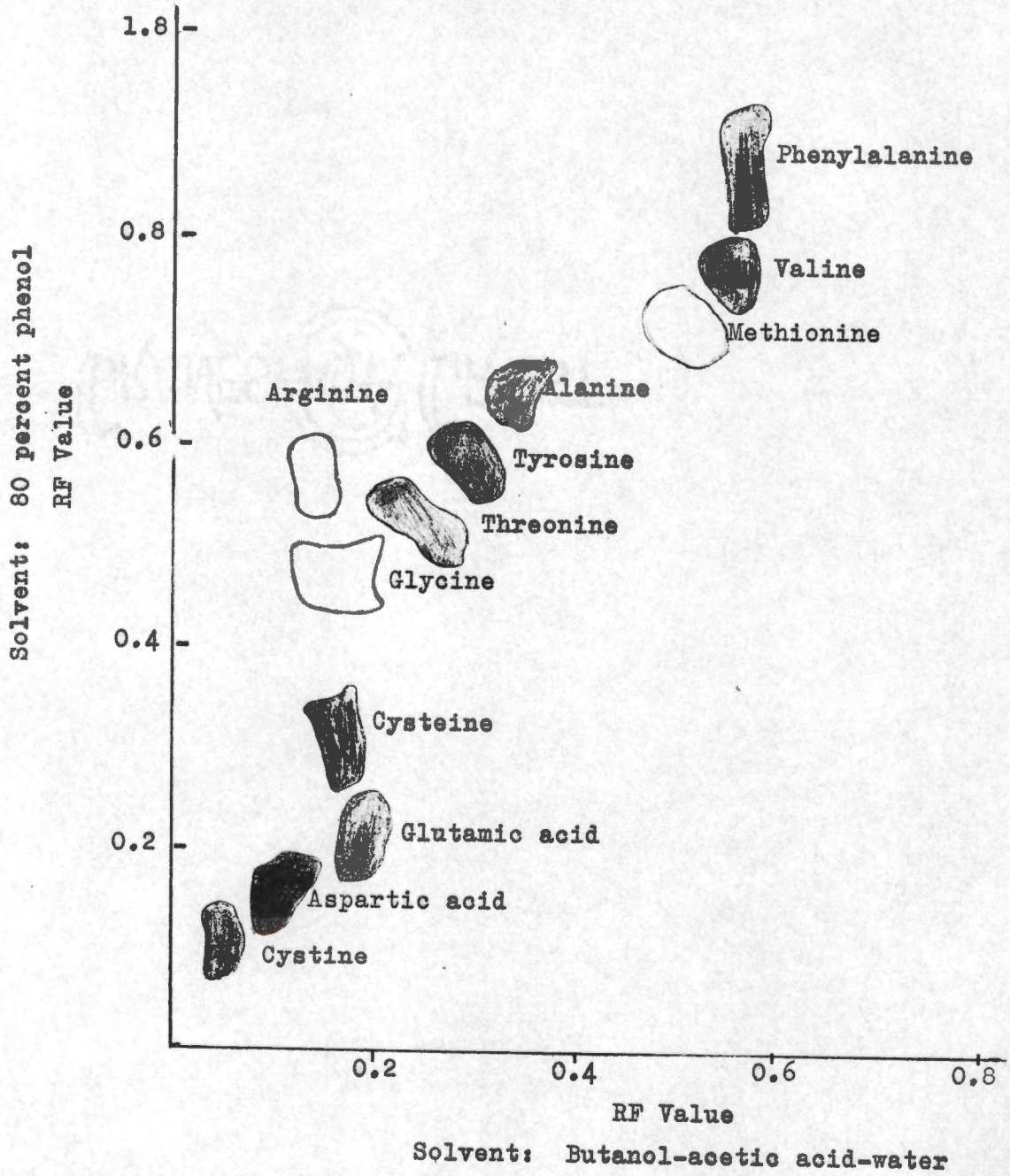

Figure 2. Free Amino Acids in Blood Serum of Infected Chicks.

evidence of reduction. By 96 hours the glycine level was completely restored.

In addition to the observed reduction of arginine, methionine and glycine no other consistent alterations were evident in the paper chromatograms. Traces of tryptophan were occasionally noticeable and serine was detected in a few isolated cases in samples from infected chicks. Chubb (1959) reported the detection of 21 amino acids from the plasma or serum of chickens and cited the failure of Ross et al. (1955a) to detect serine, leucine, lysine and histidine in particular.

The relative time and intesity of reduction in arginine, methionine and glycine and their comparative recoveries largely confirms the work of Dooley (1957) in chromatographic analysis of serum samples from day-old chicks intoxicated with S. pullorum endotoxin. In contrast, nevertheless, is the obvious ability of the surviving two-week old chick to recover from amino acid imbalance more rapidly that the one to three day old chicks.

Experiment II

The Effects of Supplementation of the Serum-Deficient Amino Acids to Infected and Noninfected Chicks

Chromatographic Analysis

The chromatograms from infected birds receiving no amino acid supplementation again revealed a marked reduction in arginine, glycine and methionine when compared with noninoculated controls at 24, 48 and 72 hours post inoculation.

Chromatograms from noninfected chicks receiving arginine supplementation exhibited a slight increase in arginine at 24 and 48 hours and a very pronounced increase at 72 hours, when compared to the noninfected and nonsupplemented control group. Chromatograms from infected chicks receiving arginine supplementation exhibited a decrease in arginine at 24 hours; a slight increase toward that of the controls at 48 hours; and at a level of recovery comparable to that of the noninoculated chicks at the end of 72 hours. Chromatograms, however, from infected birds receiving no amino acid supplementation showed a reduction in arginine which persisted at 24, 48 and 72 hours post inoculation. Arginine supplementation of infected chicks showed no apparent influence on the level of glycine or methionine in the blood serum.

Noninfected chicks receiving glycine supplementation showed moderately higher concentrations in chromatographed blood samples at 24, 48 and 72 hours when compared to control birds receiving no amino acid supplementation. Chromatograms prepared from the serum of infected birds receiving glycine supplementation demonstrated a reduction of glycine at 48 hours but no change at 24 or 72 hours when compared to nonsupplemented controls. Considering methionine depletion in the infected group with glycine supplementation, it appeared that there was at least a partial recovery of methionine by 72 hours. Previously, methionine had exhibited less recovery in the blood serum at 72 hours in nonsupplemented groups.

Chromatograms from noninfected chicks receiving methionine supplementation revealed a very sharp increase of methionine in the serum at 24 hours when compared to noninfected controls. At 72 hours the methionine concentration was still slightly higher than in nonsupplemental controls. The chromatographic response to methionine by the infected group receiving methionine supplementation was more pronounced than in either the arginine or glycine supplemented groups. At both the 24 and 48 hour periods the methionine concentration was increased well above the infected controls. At 72 hours post inoculation the chromatograms showed only a slight increase over nonsupplemented controls. Methionine supplementation did not appear to effect arginine or glycine concentrations.

Tryptophan was not successfully detected on the chromatograms prepared from the tryptophan supplemented groups. Furthermore, there were no apparent alterations on these chromatograms attributed to tryptophan supplementation.

Mortality Percent

Percent mortality for both infected and noninfected chicks and the average hour of death is shown in Table II. These data, when analyzed by analysis or variance, showed that the infected group of chicks receiving arginine supplementation had a significant decrease in mortality at the 10 percent level of probability. Mortality percent in all of the other groups was not significantly different at the 10 percent level.

TABLE II

PERCENT MORTALITY AND AVERAGE HOUR OF DEATH OF *S. PULLORUM* INFECTED AND NONINFECTED CHICKS, EXPERIMENT II

Treatment	Group	Mortality percent	Av. hour of death
Arginine	Infected	58.33[2]	12.40
Glycine	Infected	82.35	24.38
Methionine	Infected	75.00	21.41
Tryptophan	Infected	91.66	23.04
Combination[1]	Infected	75.00	16.23
Basal Control	Infected	80.50	22.97
Arginine	Noninfected	0	-
Glycine	Noninfected	2.77[3]	-
Methionine	Noninfected	0	-
Tryptophan	Noninfected	0	-
Combination[1]	Noninfected	0	-
Basal	Noninfected	0	-

[1]Includes equal portions of arginine, glycine, methionine and tryptophan.

[2]Significant at the 10 percent level of probability.

[3]Death due to nondetermined causes.

One chick died from undetermined causes in the glycine noninfected group. Macroscopic examination revealed no evidence of pathology.

Morbidity Percent

Morbidity observations revealed that, in general, by 12 hours post inoculation all chicks in the infected groups exhibited symptoms associated with pullorum disease. This was evidenced by chicks huddling together giving the appearance of being cold; labored breathing; marked loss of appetite; diarrhea; and finally death. By 24 hours post inoculation peak morbidity was observed. Comparatively, the infected group receiving arginine supplementation recovered the fastest. By 36 hours post inoculation these chicks appeared well on their way to recovery. By 48 hours the group as a whole exhibited almost complete recovery. The ability of the arginine group to recover from the initial acute septicemia was in sharp contrast to the apparent inability of the tryptophan group to recover. Only three chicks in the tryptophan supplemented inoculated group survived. Their recovery was not evident until one week after inoculation. In comparing the other infected groups to the rapid recovery of the arginine group and slow recovery of the tryptophan group, the methionine and combination group recovered moderately fast while the glycine and control group recovered moderately slow. By eight days post inoculation the chicks from all infected groups failed to exhibit signs of

morbidity. Evidence of morbidity was absent from noninfected birds at all times.

Feed Consumption

No significant differences were found in noninfected groups between treatments with respect to grams of feed consumed per chick during the two week trial period. Feed consumption per chick for the 14 day period post inoculation for each group was as follows: (1) arginine--277.3 grams; (2) glycine--254.6 grams; (3) methionine--252.6 grams; (4) tryptophan--283.2 grams; (5) combination 275.9 grams; and (6) basal control--272.7 grams.

Feed consumption records were not maintained in the infected groups due to severe mortality and limited numbers of survivors.

Average Weight Gains

The average weight gain for infected and noninfected chicks is shown in Table III. Statistical analysis showed a significant difference between the infected groups and noninfected groups ($P > 5$).[5] No significant differences were found between the various treatments within the infected or noninfected groups with respect to average weight gain per chick during the two week post inoculation.

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[5]Statistically significant at the five percent level of probability when analyzed by analysis of variance.

TABLE III

AVERAGE 2-4 WEEK WEIGHT GAIN PER CHICK POST INOCULATION, EXPERIMENT II[1]

Treatment[2]	Infection status	Gain/chick grams
Basal	Noninfected	124.75
Basal + arginine	Noninfected	116.00
Basal + glycine	Noninfected	104.12
Basal + methionine	Noninfected	114.12
Basal + tryptophan	Noninfected	125.00
Basal + combination[3]	Noninfected	125.54
Basal	Infected	68.77
Basal + arginine	Infected	60.50
Basal + glycine	Infected	92.22
Basal + methionine	Infected	84.20
Basal + tryptophan	Infected	64.00
Basal + Combination[3]	Infected	91.92

[1]Chicks two weeks of age at time of inoculation and four weeks of age at termination of experiment.

[2]Each treatment consisted of 36 chicks; three replicates with 12 chicks each.

[3]Equal amounts of arginine, glycine, methionine and tryptophan.

Straight line connects values of no significant difference at the five percent level of probability.

Experiment III

The Comparative Effects of Selected Amino Acid Supplementation and Medication of S. pullorum Infection in Chicks

Percent Mortality

The attempt in this experiment to establish an LD_{50} inoculum in the nontreated infected controls was quite successful. This provided an adequate basis for comparing mortality between groups and at the same time provided a sufficient number of survivors for evaluating other criteria.

Percent mortality for each of the 13 treatments in this trial is presented in Table IV. Data collected at each 12-hour interval showed that the peak of mortality occurred at approximately 84 hours post inoculation.

Analysis of variance showed that supplementation of the basal diet with arginine significantly reduced mortality when compared to the infected controls ($P > 5$). Methionine supplementation also showed a significant decrease in mortality. Arginine and methionine combined did not show a significant change in mortality ($P > 5$).

NF-180 in all possible combinations exhibited a significant effect on reduction of mortality ($P > 5$). The results obtained from using NF-180 as a medicant against S. pullorum infection in chicks were considered conclusive. Out of a total of 180 inoculated chicks receiving NF-180 only two chicks succumbed to the infection. This is in contrast to a 44.44 percent

TABLE IV

PERCENT MORTALITY FROM S. PULLORUM INFECTION[1], EXPERIMENT III

Treatment[2]	Mortality percent
Infected	
Basal (control)	44.44
Basal + arginine	13.33[a]
Basal + methionine	17.77[a]
Basal + arginine + methionine	40.00
Basal + NF-180	0[a]
Basal + NF-180 + arginine	0[a]
Basal + NF-180 + methionine	2.22[a]
Basal + NF-180 + arginine + methionine	2.22[a]
Basal + SEZ	13.33[a]
Basal + SEZ + arginine	2.22[a]
Basal + SEZ + methionine	15.55[a]
Basal + SEZ + arginine + methionine	20.00[a]
Noninfected	
Basal (control)	0[a]

[1]Chicks were inoculated at two weeks of age.

[2]Forty-five chicks per treatment; three replicates of fifteen chicks each.

[a]Significant difference from infected control treatment at 5 percent level or probability.

mortality in the infected controls.

SEZ resulted in a significant reduction of mortality in all cases ($P > 5$). SEZ, when combined with arginine, exerted its greatest therapeutic effect in decreasing mortality. SEZ when administered alone and arginine when administered alone both resulted in a percent mortality of 13.33 percent, however, when administered in combination resulted in a mortality percent of 2.22 percent.

No mortality occurred in the noninfected controls.

Percent Morbidity

At 12 hours post inoculation approximately 30 percent of the total number of birds appeared huddled together with ruffled feathers. No treatment differences were distinguishable at this time. At 24 hours post inoculation approximately 35 percent of all the chicks appeared affected by pullorum disease with no differences in treatment noticeable. At 36 and 48 hours there appeared an apparently greater morbidity in the infected control group, arginine supplemented group, methionine group, and arginine plus methionine group. At 60 and 72 hours post inoculation 40-45 percent of the chicks appeared severly affected by the pullorum infection. The most pronounced symptoms were huddling together, ruffling of feathers, loss of appetite, apparent emaciation and dehydration, whitish pasty diarrhea, listlessness and finally death. Total morbidity at 84 hours ranged from 50 to 60 percent and at 96 hours morbidity had risen

to an overall 65 percent with differences between treatments more obvious. The groups receiving no medication appeared the most severly effected, followed by the groups receiving SEZ and with the least severity of morbidity being evident in the groups receiving NF-180.

An apparent morbidity peak occurred with approximately 75 percent of the chicks exhibiting severe symptoms between 108 and 120 hours post inoculation. At 132 hours the chicks receiving NF-180 appeared to be recovering at a comparatively faster rate than were the chicks receiving other treatments. The chicks receiving no medication exhibited a comparatively higher morbidity in addition to a slower apparent rate of recovery. At 144 hours morbidity in all groups appeared decreasing and by 156 hours post inoculation morbidity had declined to approximately 30 to 35 percent. Percent morbidity differences between treatments at 168 and 180 hours appeared approximately 50 to 60 percent in the nonmedicated infected chicks; 35 to 40 percent in the SEZ treatments; with less than 10 percent in the group receiving NF-180.

Between 204 and 216 hours there appeared an unexplainable slight increase or peak in the morbidity level of all groups. By 240 hours post inoculation almost all birds appeared recovered, especially in the groups receiving NF-180 and to a slightly lesser degree in the SEZ group. On the twelfth day (276 hours) following inoculation all surviving chicks appeared fully recovered with no evidence of morbidity or other symptoms

associated with pullorum infection.

It was observed that the groups receiving NF-180 exhibited an ability to recover from a moribund condition the most rapidly. The group receiving SEZ seemed to recover slower than the NF-180 group but somewhat more quickly than the nonmedicated group. Data regarding morbidity showed that chicks receiving NF-180 were almost completely recovered by 168 to 180 hours post inoculation, while chicks from other groups did not reach this comparable state of recovery until 240 hours post inoculation. The non-infected control group at no time exhibited any evidence of morbidity.

Average Weight Gains

Average weight gains of chicks subjected to the 13 treatments are given in Table V. Analysis of variance was used to test for significance.

When compared to the infected controls all treatments receiving NF-180 except the arginine plus methionine combination showed a significant increase in weight gains from two to four weeks ($P > 5$). From two to four weeks of age no other significant differences were found in comparison with the infected controls.

Average weight gains of chicks from four to six weeks of age exhibited a profound equalization trend when compared to the differences between treatments that existed from two to four weeks of age. Treatments of NF-180 plus arginine, NF-180 plus methionine, and SEZ alone exhibited significantly lower average

TABLE V

AVERAGE WEIGHT GAIN OF S. PULLORUM INFECTED CHICKS BY TREATMENTS, EXPERIMENT III

Treatment[1]	Av. wt. gain per chick in grams		
	2-4 wks.	4-6 wks.	2-6 wks.
Infected			
Basal (control) (25)[2]	86.15	177.30	263.45
Basal + arginine (40)	83.23	151.20	234.43
Basal + methionine (37)	78.03	167.70	234.73
Basal + arginine + methionine (27)	74.64	146.84	221.48[a]
Basal + NF-180	138.40[a]	168.08	306.48[a]
Basal + NF-180 + arginine (45)	129.73[a]	138.66[a]	268.40
Basal + NF-180 + methionine (44)	127.77[a]	123.97[a]	251.74
Basal + NF-180 + arginine + methionine (44)	79.96	183.15	263.11
Basal + SEZ (39)	105.85	123.10[a]	228.95
Basal + SEZ + arginine (44)	106.47	159.08	265.55
Basal + SEZ + methionine (38)	86.87	167.78	254.65
Basal + SEZ + arginine + methionine (36)	71.66	175.08	257.74
Noninfected			
Basal (control)	111.06	164.74	275.80

[1]Each treatment began with 45 chicks; replicated three times with 15 chicks each.

[2]Number of survivors for each treatment indicated in parentheses.

[a]Significant difference between the infected basal control at 5 percent level of probability.

weight gains per chick during the four to six week period than did the other treatments. These two treatments receiving NF-180 had exhibited significantly higher weight gains in the previous two week period while being on medication and supplementation ($P > 5$).

The average two to six week weight gain for infected chicks was found to be significantly higher for the treatment receiving the basal diet plus NF-180 while the average weight gain for the arginine plus methionine nonmedicated treatment was significantly lower to the infected control chicks ($P > 5$).

Feed Consumption

Feed consumption in average grams per chick for both of the two week periods and the total consumption per chick for the entire study following inoculation is shown in Table VI. The first two weeks following inoculation represents the time in which medication and amino acid supplementation were administered and the last two weeks represent the time that all treatments received only the basal diet.

No significant difference between treatments was found when data were analyzed using analysis of variance ($P > 5$).

Feed efficiency for the duration of the experiment was calculated. No significant differences between treatments was found when analyzed by analysis of variance ($P > 5$).

Blood Titers

Blood titers are presented in Table VII. These values

TABLE VI

AVERAGE FEED CONSUMPTION PER CHICK BY TWO WEEK PERIOD, EXPERIMENT III[1,2]

Treatment	Grams per chick		
	2-4 wks.	4-6 wks.	2-6 wks.
Infected			
Basal (control)	308.98	521.92	830.90
Basal + arginine	350.84	436.10	786.94
Basal + methionine	309.12	461.16	770.28
Basal + arginine + methionine	279.44	479.50	758.94
Basal + NF-180	386.12	513.94	900.06
Basal + NF-180 + arginine	347.20	486.08	833.28
Basal + NF-180 + methionine	351.96	440.44	792.40
Basal + NF-180 + arginine + methionine	323.82	454.44	778.26
Basal + SEZ	305.34	418.32	723.66
Basal + SEZ + arginine	339.50	469.28	808.78
Basal + SEZ + methionine	335.30	482.06	817.36
Basal + SEZ + arginine + methionine	327.04	508.76	835.80
Noninfected			
Basal (control)	416.64	541.66	958.30

[1]Chicks were inoculated with S. pullorum at two weeks of age. Experiment was terminated at six weeks of age.

[2]No significant difference existed between treatments at the 5 percent level of probability.

TABLE VII

ANTIBODY BLOOD TITERS OF AMINO ACID SUPPLEMENT AND DRUG TREATED CHICKS, EXPERIMENT III[1]

Treatment[2]	Blood titer value[3] 0	1	2	3	4	Av. titer score
Infected						
Basal (control)	0	2	15	2	1	2.1
Basal + arginine	6	1	8	4	1	1.7
Basal + methionine	2	4	10	3	1	1.9
Basal + arginine + methionine	1	2	12	4	1	2.1
Basal + NF-180	5	7	8	0	0	1.2
Basal + NF-180 + methionine	8	5	6	1	0	1.0
Basal + NF-180 + methionine	8	0	9	3	0	1.4
Basal + NF-180 + arginine + methionine	8	3	9	0	0	1.1
Basal + SEZ	1	1	15	3	0	2.0
Basal + SEZ + arginine	0	4	10	5	1	2.2
Basal + SEZ + methionine	0	0	13	7	0	2.4
Basal + SEZ + arginine + methionine	1	3	13	3	0	1.9
Noninfected						
Basal (control)	20	0	0	0	0	

[1]Blood titers were tested at four weeks post inoculation; when chicks were six weeks of age.

[2]Twenty chicks tested from each group.

[3]Numbers listed after each treatment represent the number of chicks that were tested having these values.

represent the relative antibody blood titer at four weeks post inoculation when the chicks were six weeks of age.

The smallest number of reactors and smallest titers occurred in the four treatments receiving NF-180. Of 80 blood samples tested, 29 were found to be negative. Comparatively, the four treatments of infected birds receiving no medication showed nine negative reactors while the four treatments receiving SEZ had only two negative reactors. Furthermore, the four NF-180 treatments had only four positive reactions scoring higher than 2 in contrast with 17 in the nonmedicated groups and 19 in the SEZ group. These results point to lower average blood titers in chicks receiving NF-180.

There seems to exist no essential difference between the nonmedicated groups and the SEZ group. Among the nonmedicated treatments, however, arginine supplemented chicks resulted in lower blood titer production. Further comparisons in amino acid supplementation within the nonmedicated and medicated groups resulted in no apparent differences in average blood titer reactions due to specific amino acid supplementation.

These data suggest that NF-180 more effectively prevented or masked S. pullorum infection in the chicks than did the other treatments.

No positive reactions were found in the nonmedicated noninfected controls.

Recovery of Viable Organisms

The comparative recovery of viable S. pullorum organisms

is shown in Table VIII. A total of 37 recoveries representing 30 chicks was made from the 260 cultured chicks. From the liver 14 recoveries were made while eight were from the heart, eight from the spleen, and seven from the bone marrow. Fifteen recoveries, representing 12 birds, were made from the nonmedicated infected group. Nine recoveries, representing eight birds, were made from the groups receiving NF-180. Thirteen recoveries, representing 11 birds, were made from the groups receiving SEZ.

These data show that the treatments receiving NF-180 exhibited fewer recoveries than did any of the other groups. The greatest difference was seen in the NF-180 plus arginine treatment where no recoveries were made. Further comparison showed little difference between amino acid supplementation within the medicated and nonmedicated groups. Little difference was observed between the number of recoveries made in the SEZ group and nonmedicated group.

No recoveries were made from the noninoculated control group.

TABLE VIII

TOTAL NUMBER OF RECOVERIES FROM LIVER, HEART, SPLEEN AND BONE MARROW OF VIABLE *S. PULLORUM* ORGANISMS, EXPERIMENT III[1]

Treatment	No. of chicks with recoveries[2]	Number of recoveries				
		Liver	Heart	Spleen	Bone marrow	Total
	Infected					
Basal (control)	3	1	0	2	0	3
Basal + arginine	4	2	1	1	1	5
Basal + methionine	3	3	1	0	1	5
Basal + arginine + methionine	2	1	0	0	1	2
Basal + NF-180	2	1	0	0	1	2
Basal + NF-180 + arginine	0	0	0	0	0	0
Basal + NF-180 + methionine	2	0	1	2	0	3
Basal + NF-180 + arginine + methionine	3	1	1	1	1	4
Basal + SEZ	2	1	2	0	0	3
Basal + SEZ + arginine	2	1	0	2	0	3
Basal + SEZ + methionine	4	3	1	0	0	4
Basal + SEZ + arginine + methionine	3	0	1	0	2	3
	Noninfected					
Basal (control)	0	0	0	0	0	0
Total	30	14	8	8	7	37

[1]Chicks were examined at six weeks of age; four weeks post inoculation.

[2]Twenty chicks from each treatment were examined.

SUMMARY AND CONCLUSION

1. Three experiments were conducted using a total of 1217 two-week old Single Comb White Leghorn female chicks. Studies involved free amino acid deficiencies in the blood serum during S. pullorum infection and subsequent amino acid supplementation and medication.

2. Two-week old chicks when inoculated with S. pullorum exhibited a reduction of arginine, methionine, and glycine in the free amino acid content of blood serum. In general the peak of reduction occurred between 48 and 72 hours post inoculation. By 96 hours post inoculation chromatograms indicated an ability of the host to partially restore the depleted amino acids.

3. These data suggest that biochemical alterations do persist during the course of S. pullorum infection in chicks at two weeks of age. Oral supplementation of arginine and methionine has been shown to materially aid the host in the retention and recovery of these critical compounds during infection. Chromatographic response to glycine supplementation was moderate when compared to arginine and methionine.

4. Supplementation of chick diets with arginine and with methionine exerted a therapeutic effect against pullorum disease in reducing morbidity and mortality. Unexplained is the fact that when arginine and methionine were administered in combination no therapeutic value was established. Oral supplementation of glycine and tryptophan established no therapeutic

value against S. pullorum in two-week old chicks.

5. SEZ was found to be a desirable therapeutic agent in the control of pullorum disease. The effectiveness of SEZ appeared to be enhanced with the addition of arginine supplementation. However, treatments involving SEZ were in general inferior to those containing NF-180.

6. The administration of NF-180 was conclusive in displaying a desirable therapeutic effect against pullorum disease as evaluated by several criteria. Infected chicks receiving NF-180 exhibited decreased mortality, decreased morbidity, increased weight gains and lower antibody blood titers when compared to other groups. Arginine supplementation administered concurrently with NF-180 medication resulted in the only treatment where no viable S. pullorum organisms were recovered at four weeks post inoculation.

7. A combination of medication and supplemental arginine and methionine has been demonstrated as a promising therapeutic approach to pullorum disease in chicks in increasing effectiveness or complimenting the present incomplete method of treatment.

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APPENDIX

BASAL CHICK STARTER DIET

Ingredients	Percent
Yellow corn	63.9
Fish meal	2.5
Alfalfa meal (dehydrated, 17 percent protein)	2.5
Defl. rock phosphate	1.5
Limestone	0.6
Salt	0.48
Manganese sulfate	90 g.
Soybean oil meal (50 percent protein)	28.0
Vitamin Mix[1]	0.5

Calculated Partial Analysis:

Total crude protein	21.7
Arginine	1.4
Methionine	0.41
Cystine	0.32
Tryptophan	0.23
Glycine	1.6

[1] Vitamin mix contains: 134,187 I.U.C. of Vitamin D; 111,823 I.U. of Vitamin A; 201 mg. of Riboflavin; 0.295 mg. of Vitamin B_{12}; 20,000 mg. of Choline; 1,097 mg. of Niacin; 216 mg. of Pantothenic Acid.