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Some Aspects of Lipid Metabolism of Chicks Infected with *Salomonella pullorum*

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To the Graduate Council:

I am submitting herewith a dissertation written by Charles Dean Jeffries entitled "Some Aspects of Lipid Metabolism of Chicks Infected with *Salomonella pullorum*." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Biochemistry and Cellular and Molecular Biology.

D. F. Holtman, Major Professor

We have read this dissertation and recommend its acceptance:

J. Orvis Mundt, John M. Woodward, Frank E. Staudt, Samuel R. Tipton

Accepted for the Council:
Dixie L. Thompson

Vice Provost and Dean of the Graduate School

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

July 31, 1958

To the Graduate Council:

I am submitting herewith a thesis written by Charles Dean Jeffries entitled "Some Aspects of Lipid Metabolism of Chicks Infected with 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.

Frank Holtman
Major Professor

We have read this thesis and recommend its acceptance:

J. Orvin Mundt

John M. Woodward

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Accepted for the Council:

Dale Hawthorne
Dean of the Graduate School

SOME ASPECTS OF LIPID METABOLISM OF CHICKS INFECTED WITH *SALMONELLA PULLORUM*

A DISSERTATION

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

Charles Dean Jeffries

August 1958

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C. D. J.

TABLE OF CONTENTS

LIST OF TABLES

TABLE PAGE

LIST OF FIGURES

FIGURE PAGE

I. INTRODUCTION

The literature concerning the pathology accompanying infectious diseases has concerned, predominantly, gross and microscopic pathology. The biochemical changes in pathologic conditions have had little attention except for the generally accepted clinical procedures. Within the last decade, however, a change in emphasis has begun. A number of investigators have reported studies of enzymatic changes observed in infected animals. Many of these observations, however, have been with unnatural host-parasite systems.

The desirability of using a natural host-parasite system is obvious. Among the Enterobacteriaceae there are a limited number of complexes which are suitable for study with experimental animals. One of these is the well documented pullorum disease, a malady which has been accepted as being specific for chicks.

The etiologic agent has been known for over half a century, during which the epidemiology and control methods have been worked out in detail. The biochemical pathology has, on the contrary, been studied in detail only in recent years.

Reports from this laboratory have described alteration in nitrogen and carbohydrate metabolism. Chromatographic procedures have shown qualitative changes in amino acids occur in the disease. Nitrogen excretion is altered as demonstrated by the high levels of urea in the blood of normally uricotelic animals. The carbohydrate studies have been concerned largely with the components of the Krebs cycle. This has been accomplished by enzyme studies, assay of the concentration of inter-

mediates and by using inhibitors of enzyme activity.

Many of these alterations were similar to those encountered in carbon tetrachloride poisoning. One of the most characteristic results of poisoning with carbon tetrachloride is deposition of fat in the liver. Such a change has been observed in histologic preparations of liver from chicks infected with Salmonella pullorum. This and the fact that methionine, an amino acid which exerts a marked lipotropic effect, disappears in the diseased bird led to the study of changes in the lipids which is reported in this thesis. This study has been correlated, in part, with previous studies of amino acid therapy. Certain lipotropic agents have been used similarly in order to compare therapeutic effects and to arrive at a cause for the variations in the fat from the liver.

II. HISTORICAL REVIEW

Salmonella pullorum was first isolated in 1899 from chicks involved in an epizootic in Indiana. Rettger (1900) subsequently reported the disease to be discrete from other diarrheal diseases of fowl. A second paper (Rettger, 1901) described an epizootic observed in 1900 which Rettger believed to be more severe than the first. In each case the causative organism was isolated on nutrient agar plates and the physiologic reactions determined. The organism was proven to be the pathogen by fulfilling Koch's postulates.

Even though Rettger had observed the disease and isolated the organism in 1899, he allowed the taxonomy to remain obscure until 1909. At this time he recommended it be called Bacterium pullorum, the name being suggested incidental to observations concerning the symptoms of the disease. It was noted that the yolk of chicks infected with S. pullorum was not absorbed and that organisms could readily be isolated from the unabsorbed yolk (Rettger, 1909).

During the same period Rettger and Stoneburn (1909) at the Storrs, Connecticut Experiment Station had been investigating the epidemiology of the disease. As a result of this work they described a cycle of infection which found wide acceptance. It was reported that the infected hen could pass infected eggs, some of which might hatch and give rise to the infected chicks. These could infect normal chicks, or some might survive to adulthood and serve as carriers. Another source of infection was the contaminated incubator in which the organism is transmitted on the down and dust. The most dangerous source of infection is probably

to be found in picking for food on contaminated range or drinking contaminated water.

Weldin and Weaver (1930) showed definitely that the disease could be spread between chicks by oral administration of infected fecal material to normal chicks. The chronic carrier state of the adult hen was established when it was found that S. pullorum could be recovered in large numbers from the feces of apparently healthy hens (Kerr, 1930).

This cycle has been further investigated by a number of investigators. The yolk was found to be an important source of the organism when carrier hens were in the flock (Rettger and Stoneburn, 1909; Runnels and Van Roekel, 1927a, b; Weaver and Weldin, 1931). The organism, however, could not be isolated with regularity from eggs laid by reactors, nor was there a correlation with the percentage of infected eggs laid. A breed variation in susceptibility to S. pullorum was indicated in these studies.

It is interesting to note that infection with S. pullorum does not affect the fertility of the egg, but the embryos in infected eggs often die before hatching. Those which survive to hatching usually contract pullorum disease or at least manifest symptoms at a later date (Beaudette, et al., 1923a, b). The mortality of pullorum disease has been reported to be heaviest during the first three weeks following hatching. The severity of the disease diminishes rapidly after this time (Hamilton, 1932). Though there are organisms in the egg the effect of these may be negated by the presence of agglutinins in the albumin. Beaudette (1932) found that normal and reacting hens could be separated

on this basis. Later, Buxton (1952) reported finding antibodies against S. pullorum in the yolk of eggs from vaccinated hens. The antibodies were detectable in the serum of the chick at hatching, but decreased in the first three days after hatching.

Jeffries and Holtman (1958) have reported the growth of S. pullorum in allantoic fluid of embryonated eggs to be logarithmic, and that small numbers of organisms were required to establish a fatal infection. The bacterial population was relatively stable at the time of death, i.e., about 10^9 organisms per gram of allantoic fluid.

Gilfillan, et al. (1956a) have reported a rapid increase in the number of organisms in the blood and carcass of chicks infected with S. pullorum. Similar results have been reported by Berry (1955) for mice infected with S. typhimurium.

Wolf (1958) found S. pullorum to increase in the spleen, kidney, liver and heart in chicks infected one day after hatching. Deutectomy affected the numbers of organisms found, the number being lower in the deutectomized bird than in the normal. This suggests that the yolk plays a role in the infection, possibly serving as a reservoir for the parasite in which natural defenses may be non-existent. Indeed, the organism reproduced more rapidly in the yolk than in the tissues investigated.

The symptoms associated with pullorum disease are neither specific nor individual for this disease. Chicks affected tend to be somnolent, weak, have a loss of appetite and in the most severe cases death results. Chicks that die in the early stages of brooding seldom show many lesions or at most only very limited lesions. The liver is enlarged and congested

and the normal yellow color may be streaked with hemorrhages. The yolk sac usually reveals slight or no alteration, but in protracted cases an interference with absorption may occur. There have been reports of change in consistency in such cases as well (Van Roekel, 1952).

The histologic changes do not serve to differentiate pullorum from other diseases of young poultry. The microscopic changes in the liver are hyperemia, hemorrhages, focal degeneration and the beginnings of leucocytic infiltration. Fatty and albuminous degenerative changes are found in dead chicks, but also may be observed immediately prior to the time of death (Doyle and Mathew, 1928).

The histologic changes observed indicate that modification of liver activities would occur. Because the histologic changes are not especially marked until about the time of death a suggestion that the derangement of metabolic functions occurs earlier has been made. The importance of alteration of the physiologic activities of the liver, a most active metabolic site, has been emphasized (Popper and Schaffner, 1957). Changes in the liver metabolism of chicks infected with S. pullorum have been observed. These observations dealt with nitrogen metabolism, among them amino acid changes and the alteration in the nitrogen excretion patterns. Modifications of the carbohydrate metabolism have been observed, especially with relation to the Krebs cycle.

A change in the free amino acid profile of the blood and liver when chicks are infected with S. pullorum has been demonstrated (Ross, 1956; Ross et al., 1955a; Dooley, 1957; Dooley, et al., 1957, 1958a). Sixteen amino acids appeared on the chromatographs of the deproteinized

serum or of the deproteinized liver homogenate from normal chicks. However, upon chromatographing these substances from the infected bird four amino acids, arginine, methionine, glycine and tryptophan, were found to be missing. This qualitative change is apparent in both the free and the bound forms, the latter being demonstrable in hydrolyzates. A similar response was manifested when the chicks were intoxicated with the endotoxin from S. pullorum (Dooley, 1957).

This information has been made use of in the therapy of the disease with a degree of success (Ross, et al., 1955b; Dooley, 1957). Arginine was found to be particularly effective and methionine slightly so. Glycine and tryptophan were only slightly active in alleviation of the disease.

In further studies it was found that the chick, which excretes nitrogenous wastes principally as uric acid, has a markedly increased blood urea level when infected with S. pullorum. When treated with arginine the urea levels increased dramatically (Ross, et al., 1956). These data were used as a basis for the hypothesis that in the diseased chick there is a reversion to an evolutionary metabolic cycle. Needham, et al. (1935) have shown that the embryonic chicken possesses an active arginase and as a result excretes a large proportion of its nitrogenous waste as urea. The maximum activity of this enzyme occurs at the ninth day of incubation. Clementi (1946) subsequently could show no arginase activity in the chick after hatching. Ross (1956) has shown this enzyme and consequently the Krebs-Henseleit (ornithine-citrulline) cycle to be activated in the course of pullorum disease. As a result of this observation the effectiveness of the other members of the cycle were investi-

gated. Administration of these amino acids exhibited a protective effect through extension of the survival time. However, this treatment was not satisfactory as the mortality was greater in the treated chicks than among the infected chicks.

Other changes in nitrogen metabolism have been noted, among them an increase in blood ammonia. During infection the increase was found to occur both posterior and anterior to the liver, although the vena cava blood carried much less ammonia (Dooley, 1957). Administration of arginine led to a decrease in the ammonia level in the diseased bird, but to a slight increase in the normal. In this case the change effected by administration of the arginine apparently was found in the synthesis of urea as reported by Ross, et al. (1956). Changes in creatine levels have been noted and were observed to rise about as urea did. This observation led Dooley (1957) to postulate an alternate pathway to be acting in conjunction with the Kreb-Henseleit cycle during the infection. The amidine group of arginine may be transferred to glycine yielding guanidoacetic acid and ornithine. The guanidoacetic acid is methylated to yield creatine which can be dehydrated to yield the internal anhydride, creatinine, a nitrogenous excretion product. A summary of the interrelationship of these various nitrogenous substances was developed by Dooley (1957) and Dooley, et al. (1958b).

Cystine was reported by Gilfillan, et al. (1955) to be the only amino acid essential for growth of S. pullorum, CDC 3522/51, on synthetic media. Therefore, it is improbable that a definite relationship exists between growth of the organism and the disappearance of the amino acids in the infected chick. Arginine was found to stimulate growth of

S. pullorum to some extent, and it may be utilized by the parasite in the infection.

Similar responses have been observed in another system. Woodward, et al. (1954) found a loss of free amino acids in the serum of rats infected with Pasteurella tularensis. These observations led to the determination of the effect of this disease on the metabolism of amino acids. It was found that the amino acid oxidase of liver decreased in activity and the glutamic-oxaloacetic transaminase activity was not particularly affected (Mayhew, 1954; Woodward and Mayhew, 1956). The glutamic-oxaloacetic transaminase activity of the serum in this disease has been found to be increased rather markedly (Pooley and Woodward, 1957).

Studies concerned with the carbohydrate metabolism have been carried out by Gilfillan (1956). Dooley (1957) made similar studies to determine the effect of the endotoxin on the same activities.

Gilfillan, et al. (1956a) and Gilfillan (1956) reported the use of the Krebs cycle inhibitors, sodium fluoroacetate, sodium arsenite and malonate, and intermediates, citrate and succinate, in modification of pullorum disease in young chicks. These substances were shown to decrease survival time of the infected animals, as well as to allow organisms to multiple more rapidly. Berry (1955) and Berry and Mitchell (1954) have made similar observations with mice infected with S. typhimurium. Berry (1955) found the numbers of the parasite to be about the same at the time of death, whether the animal had been treated in this manner or not; however, death occurred sooner in the treated mice.

Studies on the level of citrate in the chick infected with S. pullorum were investigated by Gilfillan, et al. (1956b). Citrate was found to increase as the infection progressed, whether the chick had been poisoned by injection of Krebs cycle inhibitors or not. In case of poisoning the increase occurred much sooner. Dooley (1957) found the citrate levels to be increased after intoxication of the chick with the endotoxin of S. pullorum.

Berry and his associates (Berry and Mitchell, 1953a, b; Berry et al., 1954b, 1956) have investigated the effect of Krebs cycle inhibitors and intermediates on the infection of mice with S. typhimurium. The data presented in these papers are in agreement with the observations of Gilfillan quoted above. Berry, et al. (1954b) found that the Krebs cycle inhibitors and intermediates were also capable of increasing the susceptibility of mice to infection with organisms other than the Enterobacteriaceae. The effectiveness of each compound varied with the test organism.

To further elucidate the problem of infection and its effect on carbohydrate metabolism the activity of several enzymes were assayed (Gilfillan, et al., 1956b). It was found that the enzyme systems in which the pyridine nucleotides are involved were decreased in activity as the infection progressed. The systems studied were aconitase, fumarase, and lactic, malic and isocitric dehydrognases. In addition, the activity of succinoxidase was decreased. Succinoxidase has been found to be susceptible to endotoxin intoxication in rabbits (Kun and Miller, 1948) and in guinea pigs (De Barbieri and Scevola, 1956).

Dooley (1957) found blood sugar to be reduced by infection as

well as endotoxin, an observation which is supported by the literature (Kun and Miller, 1948). Cameron, et al. (1958) have reported the glycogen content of livers from the chick infected with S. pullorum to be reduced as determined by infrared spectrophotometry.

Many of the observations made by Gilfillan (1956) concerning the carbohydrate metabolism are similar to those observed in carbon tetrachloride poisoning. Zollner and Raisch (1956) have found the succinoxidase activity of rat liver homogenate to be decreased within ten hours following administration of carbon tetrachloride. A similar response was observed in mice (Zollner and Groebl, 1952).

Dianzani and his colleagues have found a number of changes associated with carbon tetrachloride poisoning of rats. A decrease in the amount of diphosphopyridine nucleotide has been reported (Dianzani, 1955), however, addition of DPN returned to normal the activity of enzymes requiring this co-factor. Adenosine triphosphate is decreased and adenosine diphosphate increased in poisoning with carbon tetrachloride (Dianzani, 1957). These changes have been observed to occur prior to histologic change.

Other changes which have been observed in carbon tetrachloride poisoning (Eden, et al., 1954) and in infection with S. pullorum or intoxication with its endotoxin (Dooley, 1957) is an increased creatine level. Severi and Fonnesu (1956) have reported the amount of coenzyme A in fatty liver induced by carbon tetrachloride was reduced, a finding similar to that reported by Dooley, et al. (1957) for chicks infected with S. pullorum or those receiving the endotoxin from this organism. Phosphatase activity of the plasma is increased, probably due to a loss

of the ability by the liver to absorb excess phosphatase (Talageri, et al., 1951).

Glutamic-oxaloacetic transaminase increased following exposure to an atmosphere containing carbon tetrachloride (Block and Cornish, 1958) as well as in rats infected with P. tularensis (Pooley and Woodward, 1957).

Formal, et al. (1958a) found that guinea pigs could be infected fatally with Shigella flexneri after inducing a fatty liver by starvation. Formal, et al. (1958b) observed that similar results can be obtained by poisoning the guinea pig with carbon tetrachloride prior to administration of the infecting organism.

The chick has been shown to have hyperlipemia and a fatty liver at the time of hatching. One week after hatching the lipids fall to about one-fourth the level at hatching (Entenman, et al., 1940). The level of lipid is above that present in chickens for about four weeks after hatching.

The hyperlipemic nature of the chick may play an important role in determining the susceptibility to infection with pullorum disease. This in conjunction with the similarity of the effect of carbon tetrachloride poisoning on various animals and infection of chicks with S. pullorum suggested the importance of the lipid metabolism.

Christie and Judah (1954) have found that administration of choline early in carbon tetrachloride poisoning will delay the appearance of the symptoms but not prevent them. This action was not explained. Choline has long been known to be a lipotropic agent of merit (Best, 1950, 1956). A number of other agents have been demonstrated to

exert a similar effect. A very important requirement is the availability of labile methyl groups such as are encountered in betaine, lipocaic and methionine. Eckstein (1952) and Tucker and Eckstein (1937) reported methionine to be the only amino acid which exerted a lipotropic effect in choline deficient diets. In the latter paper cystine was reported to be lipogenic. Singal, et al. (1953a, b) have, on the contrary, found threonine to exert a lipotropic effect if small amounts of choline are present in the diet. In the absence of threonine large amounts of choline prevented fatty infiltration. Methionine in large amounts also overcame the threonine and choline deficiency. These authors suggested the existence of different metabolic requirements for threonine and choline. Incorporation of phosphorus into the phospholipid and nucleic acid was found to be reduced in animals which were fed a threonine deficient diet.

Phospholipid has been assigned, by a number of investigators (Dawson, 1957), an important role in the maintenance of structural integrity of the cell and its various organelles, principally the mitochondria (Christie and Judah, 1954). The loss of phospholipid would be expected to lead to a loss of metabolic activity and this has been demonstrated following treatment of mitochondria with phospholipase (Denstedt, 1956). The loss of activity of such oriented enzymes has been demonstrated in previous experiments in this laboratory (Gilfillan, 1956; Gilfillan, et al., 1956b) which would indicate a disruption of the positioning of constituent enzymes. An injury of some other sort such as inhibition of a step in metabolism may be responsible for the alterations observed such as the administration of enzyme inhibitors

(Gilfillan, et al., 1956b).

The production of phospholipid is a metabolically expensive process (Kennedy, 1957) and may in part account for the appearance of larger amounts of inorganic phosphate in the serum of infected chicks (Dooley, 1957). On the other hand, methylation of guanidoacetic acid to creatine is similarly an expensive process and as demonstrated by Dooley (1957) must exert its influence in the course of pullorum disease.

The phospholipids have been implicated in the transport of lipid from the liver to depots (Popper and Schaffner, 1957); however, this concept of the function of the phosphatids has become untenable (Dawson, 1957), nevertheless they may play a role in maintaining the colloidal dispersion of fat droplets. Zilversmit, et al. (1948) have suggested the phospholipid plays a role in the metabolism of fat.

It has been demonstrated that the various tissues are capable of synthesizing the phospholipids. Hevesy, et al. (1938) established that following injection of radioactive phosphate into the yolk of embryonated eggs little or no activity appeared in its phospholipid. They found the lipid extracted from the embryonal tissue showed appreciable activity, and consequently made the suggestion that the phospholipid is synthesized entirely from component parts. Therefore, the chick must expend an appreciable amount of energy to maintain the structural integrity of the cell and its inclusions.

The dietary state of the host has been demonstrated, although not without controversy, to affect the susceptibility to infection. It is possible to establish an infection with Shigella flexneri in

starved guinea pigs, a disease to which these animals are normally resistant (Formal, et al., 1958a). Mice varied in their resistance to infection according to the diet which they were being fed (Schaedler and Dubos, 1956; Dubos and Schaedler, 1958). Miles (1951) in a series of studies designed to determine the effect of diet upon complement and agglutinin titer found no definite change, but did report a loss of natural resistance to bacterial infection. To the contrary, Metcoff (1949) reported protein deficiency did not alter susceptibility nor resistance. No physiophathologic response was observed by that investigator in animals fed a nutritionally deficient diet. Complement has been reported to be affected by carbon tetrachloride and ethionine, however, it was not affected in fatty liver induced by feeding a high cholesterol diet. Therefore, Rice (1954) concluded that some condition other than fat deposition was responsible for the loss of complement.

Therefore, it can be said that the proper supply of adequate protein is essential for the well being of the host. Corkill (1950) has enumerated conditions which lead to a depletion of metabolic stores with a subsequent disturbance of a host-parasite balance. He also points out the defective quality of plant protein for animal diets because of the lack of or short supply of essential amino acids and therefore, the need for some animal protein in the diet. Robertson and Doyle (1936) have reported similar findings for rats infected with S. typhimurium, and Ritterson and Stauber (1949) for hamsters infected with Leishmania donovanni.

Many of the changes which occur during pullorum disease are similar to those which take place in toxic fatty infiltration of the

liver. The newly hatched chick is hyperlipemic and has a fatty liver, therefore, additional metabolic disturbances which occur in pullorum disease, such as a decrease in methionine, may lead to further changes in lipid distribution. Alteration of the Krebs cycle activity would affect the fat composition. It was proposed to investigate the relationship of lipid to the pathogenesis of the disease and to determine the effect of amino acid therapy on lipid metabolism of the infected chick.

III. MATERIALS

1. The organism

A strain of Salmonella pullorum (3522/51; NJ 1-40127; XII_2) obtained from the Communicable Disease Center, United States Public Health Service, Chamblee, Georgia was used throughout this investigation.

On S-S agar the colonies were small, colorless, transparent and possessed an entire margin in 24 to 36 hr at 37 C. On brilliant green agar, colonies appeared bright red, as was the surrounding area. In smears from a turbid growth in nutrient broth, organisms appeared as short, gram negative rods, 1 to 2 μ in length. In hanging drop preparations, the organisms were non-motile. On nutrient agar plates, the colonies were grayish white, smooth, glistening, and entire to undulate. This strain produced acid when inoculated into rhamnose broth and produced both acid and gas from xylose, dextrose, and mannitol. No change was observed on maltose, lactose, dulcitol and salicin. Indol was not formed, urea was not hydrolyzed, and citrate was not utilized. Hydrogen sulfide was formed.

Chick passages were made periodically to insure maximum virulence. Upon isolation of S. pullorum by streaking from infected animals to S-S agar (Difco), isolated colonies were transferred to trypticase soy agar slants (BBL) and stored at 5 C.

2. The experimental animal

One to two day old White Leghorn cockerels (Babcock strain), hatched from flocks which had tested "pullorum free", were used in all

experiments. Chicks of 35 ± 1 gm were selected at random and grouped for experimentation. Food and water were made available, ad libitum, 18 hours after inoculation. The feed was an antibiotic free commercial ration which supports satisfactorily growth of normal chicks.

3. Chemicals

The amino acids and lipotropic agents used in this study were obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio. All were designated as chemically pure.

The solvents used for lipid extraction were chemically pure and were used without further purification.

The radiophosphorus used to determine the rate of phospholipid synthesis was obtained from the Oak Ridge National Laboratories as carrier-free orthophosphate. Prior to use the solution was adjusted to contain 10 μc per ml.

The alcohol used in saponification was purified by standing over potassium hydroxide and aluminum for several days after which it was redistilled. The potassium ethanolate was prepared by dissolving about 3.5 gm of CO_2 free KOH in 100 ml redistilled 95 per cent ethanol. This was diluted with nine parts redistilled ethanol on the day it was to be used.

All other chemicals were reagent grade.

IV. METHODS

1. The inoculum

Cell suspensions used in preparing the inocula were made by suspending the growth from 24 hour Trypticase Soy (BBL) agar slants (1.5 per cent agar added to broth) in sterile water. All suspensions were adjusted in the Klett-Summerson photoelectric colorimeter with filter #66 to give a reading of 115 to 125. A suspension prepared in this manner had an average of 8.9×10^8 bacteria per ml. From this suspension ten-fold dilutions were prepared and the 10^{-5} dilution used exclusively for inoculation of the chicks.

The infecting dose was contained in 1 ml and the chick was injected intraperitoneally with this volume. All inocula were assayed by use of the spread plate method on S-S agar. The plates were counted after 24-48 hours of incubation in order to obtain an estimate of the number of organisms injected.

2. Treatment with amino acids and lipotropic agents

The solutions of arginine, methionine, threonine and inositol were prepared by dissolving 50 mg in 1 ml sterile distilled water for each bird to be treated. Betaine, due to its toxicity, was administered at 10 mg per bird and prepared as above. These solutions were used immediately after preparation without further sterilization.

The treated chicks received a single injection of 1 ml of the therapeutic agent 36 hours after receiving the infecting dose. In those cases in which a treated, normal control was used the therapeutic dose was administered at the same time as that of the treated, infected birds.

3. Lipid Extraction

Approximately equal amounts of liver from either three or five chicks were pooled. The final weight amounted to about 900 mg in the former case and to 1500 to 2000 mg in the latter. The pooled tissue was homogenized in 5 ml of distilled water and to this 10 ml of a mixture of three parts ethanol and one part ethyl ether were added and the suspension of tissue further homogenized.

The homogenate was treated in one of two ways at this point. In one series of experiments 5 ml aliquots were transferred to 50 ml centrifuge tubes. To each 10 ml of 3:1 ethanol-ether was added. In the second procedure two sets of tissue were weighed and homogenized in 5 ml distilled water and further homogenized after addition of 10 ml 3:1 ethanol-ether. The total volume of homogenate was transferred to a centrifuge tube and the homogenizer tube washed twice with 5 ml 3:1 ethanol-ether.

The homogenates were extracted three times with 3:1 ethanol-ether and twice with ethyl ether (table 1). The pooled solvent washes were concentrated by boiling, the final volume being about 10 ml. This was transferred to centrifuge tubes quantitatively and the lipid re-extracted in four washes with petroleum ether (30-60). The fraction soluble in petroleum ether was aspirated into tared 125 ml Erlenmeyer flasks (Herrman, 1957) and the solvent evaporated *in vacuo*. The lipid residue was weighed and used for further study.

4. Infrared spectrophotometry

These studies were carried out by the technique of Stimson and O'Donnell (1952) and Schiedt *et al.* (1952) with slight modification.

TABLE 1

Procedure for Extraction of Total Lipid from Homogenized Liver

Homogenize about 1.5 gm liver in 5 ml distilled water. Add 10 ml 3:1 ethanol:ether and homogenize.
Wash homogenizer tube with two 5 ml portions of 3:1 ethanol:ether.
Hold 1 hr at 60 C or overnight at about 25 C and 30 min at 60 C.
Centrifuge at 2,000 RPM for 15 min. Aspirate supernate into flask.
Re-extract with 25 ml 3:1 ethanol:ether at 60 C for 1 hr.
Centrifuge at 2,000 RPM for 15 min. Aspirate supernate into flask.
Re-extract precipitate with 15 ml 3:1 ethanol:ether at 60 C for 30 min.
Centrifuge at 2,000 RPM for 15 min. Aspirate supernate into flask.
Extract precipitate with 15 ml ethyl ether. Heat to boiling.
Centrifuge at 2,000 RPM for 15 min. Aspirate supernate into flask.
Extract precipitate with 15 ml ethyl ether. Allow to stand at room temperature for 10 min.
Centrifuge at 2,000 RPM for 15 min. Aspirate supernate into flask. Discard precipitate.
Concentrate contents of flask to about 10 ml by boiling.
Re-extract with four washes of petroleum ether (30-60 C b.p.) totaling 70 ml. Discard lower fraction.
Evaporate the solvent *in vacuo* at room temperature.

The spectra from 2.5 to 15 μ were recorded on a Perkin-Elmer model 137 spectrophotometer ("Infracord").

One mg of the lipid was dissolved in 1 ml of carbon tetrachloride, and 1 ml of this solution was added to 199 mg finely ground KBr and mixed well. The solvent was evaporated under a stream of nitrogen, and the lipid-salt mixture was triturated before the pellet was pressed. A reference pellet was prepared in a similar manner with only carbon tetrachloride in order to cancel any interference caused by this compound or impurities.

5. Saponification

The lipid residue was weighed and 1 ml of petroleum ether added to redissolve it. Twenty-five ml of potassium ethanolate were added and the flasks were tightly covered with aluminum foil. The flasks were heated to boiling on an electric hot plate and then held at a gentle boil for one half hour.

The saponified solution was allowed to cool slightly, 1 ml of 1 per cent phenolphthalein added, and the unused KOH determined by back titration with HCl.

6. Radiophosphorus studies

The chicks used in this study were treated in every respect as for the other data reported except that 1 μc of P^{32} as inorganic phosphate was administered 4 hr prior to slaughter.

The lipid was extracted according to the procedure outlined above and 50 mg of the lipid residue plated on stainless steel planchets for counting. Counts were made with thin end window Geiger-Muller tube

(1.4 mg/cm^2) and decade scaler (Chicago-Nuclear Corporation Model 151). The sample to tube distance was 1 cm. The counting time was 3 min, the background was subtracted and the count adjusted to counts per minute.

V. RESULTS

1. The infrared spectra of liver lipid

The infrared spectra of the total lipid from the normal and infected chicks showed no discernible differences (figure 1). Such differences as occurred were too subtle to distinguish in the mixtures obtained by the extraction procedure used in this study.

It was possible to discern certain important characteristics such as the ester bond by peaks at 5.8 μ and 8.5 μ. Those peaks may be observed in the spectrum obtained from lecithin (figure 2). Cholesterol (figure 2) apparently accounted for few of the characteristics observed in the spectra of the total lipid sample. On the other hand, the spectrum of the non-saponifiable fraction corresponded almost exactly with that of cholesterol.

Some changes were observed with increasing age of the chick. These changes were in the range of 7.8 to 9.5 μ (figure 3). A similar change was observed in the infected chick.

2. Saponification values of liver lipids

The saponification value of the lipid extracted from the normal chick showed a general tendency to increase as the bird aged (figures 4, 7, 8, 9). Some differences in these values were noted between experiments.

In the infected chick there was an initial increase which was rather rapid with relation to the normal. A peak was reached, the hour of which varied, and then a decline to below the initial value followed (figures 5, 7, 8, 9).

Chicks treated with methionine showed a slight initial decline in saponifiable lipid after which the value returned to normal (figure 4). In the infected chick the decline in saponifiable lipid was far more dramatic than that encountered in the normal (figures 6, 7). The decline was followed by a temporary rise and then a further decline.

The effect of betaine was about the same as that obtained with methionine, despite the fact that less of the former was used (figure 7). Even at the level used, betaine was moderately toxic for chicks. The effect of arginine and threonine (figure 8) was quite dramatic with a very marked decrease in saponifiable lipid which was followed by a rapid increase in lipid levels. Arginine was more effective than inositol in reducing the saponifiable lipid in the infected chick (figure 9). Arginine also exerted its effect over a longer period of time than did inositol, but inositol was more effective than arginine in the initial period of the observation.

3. Phospholipid synthesis

The incorporation of radioactive phosphate into the lipid fraction of the liver occurred at an appreciable rate. The normal chick showed little variation in metabolism during the period of these experiments and the count had a tendency to increase as the chick aged (table 2, 3).

The infected chick initially had a markedly reduced count which was followed by a marked increase in the activity of the lipid (table 2). In a second experiment the activity of the lipid from infected

liver was higher than normal. This sample (table 3) rose to a peak and then fell.

Treatment of the chicks by administration of methionine reduced the amount of radioactive phosphorus incorporated into the lipids of the liver of both the normal and the infected chick (table 2). Administration of methionine to the infected chick held the activity of the phospholipid at the same level as the treated normal.

Arginine (table 3) effected a decrease in the activity of the lipid in the infected chick following intraperitoneal injection. This amino acid gave about the same results when given to the normal chick.

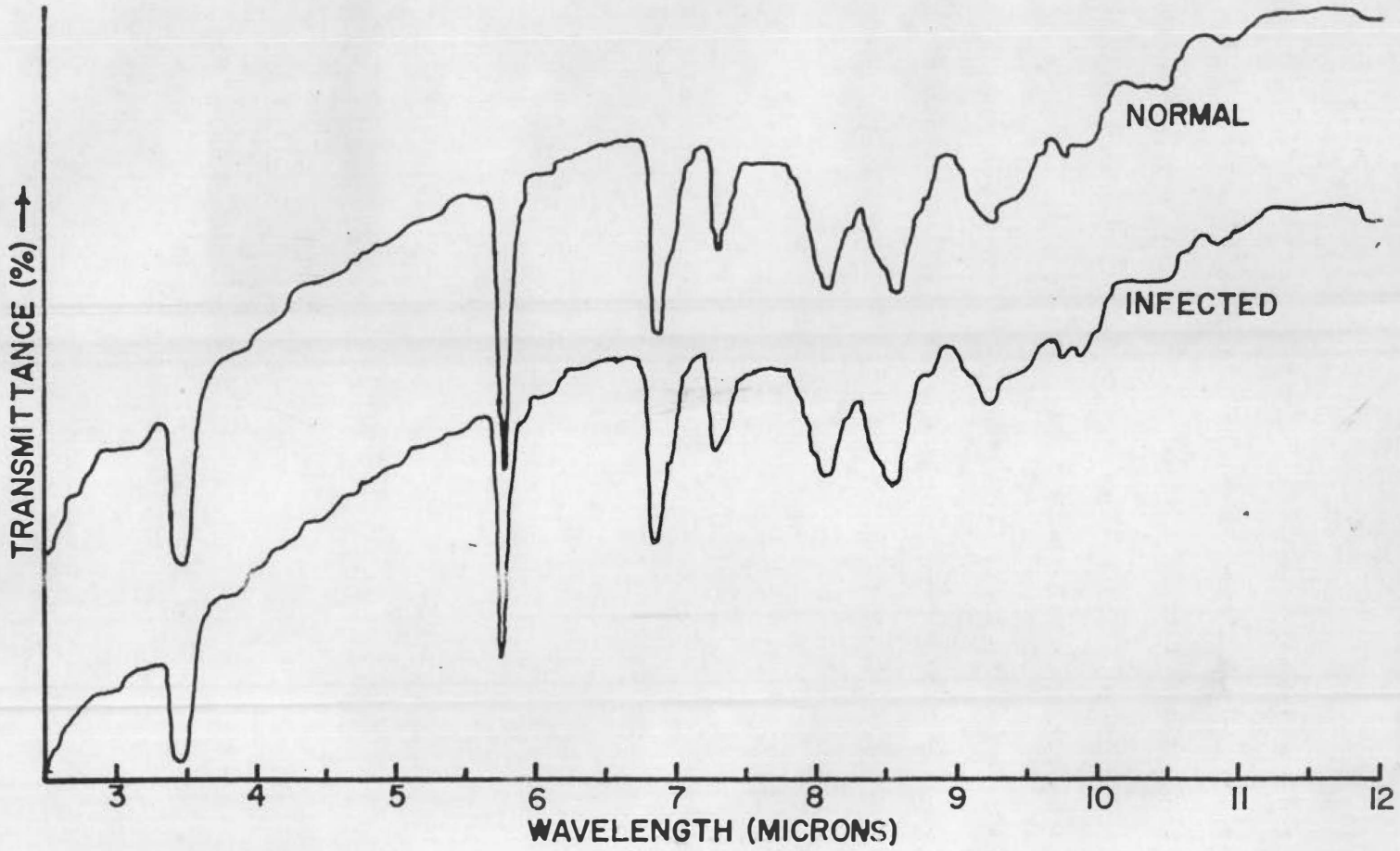

Figure 1. Infrared absorption spectra of the lipid extracted from livers of normal and of infected chicks.

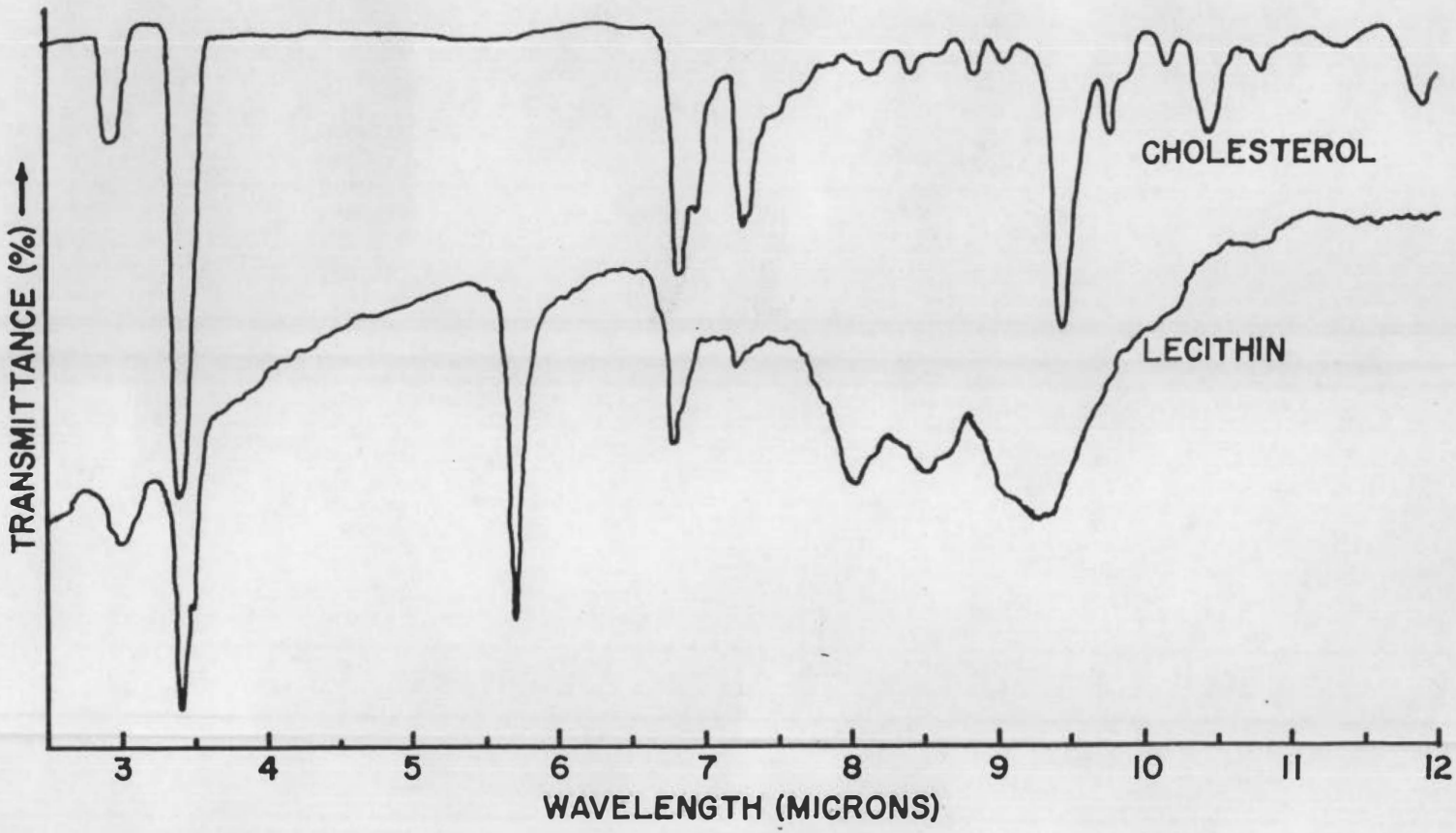

Figure 2. Infrared absorption spectra of commercial lecithin and cholesterol.

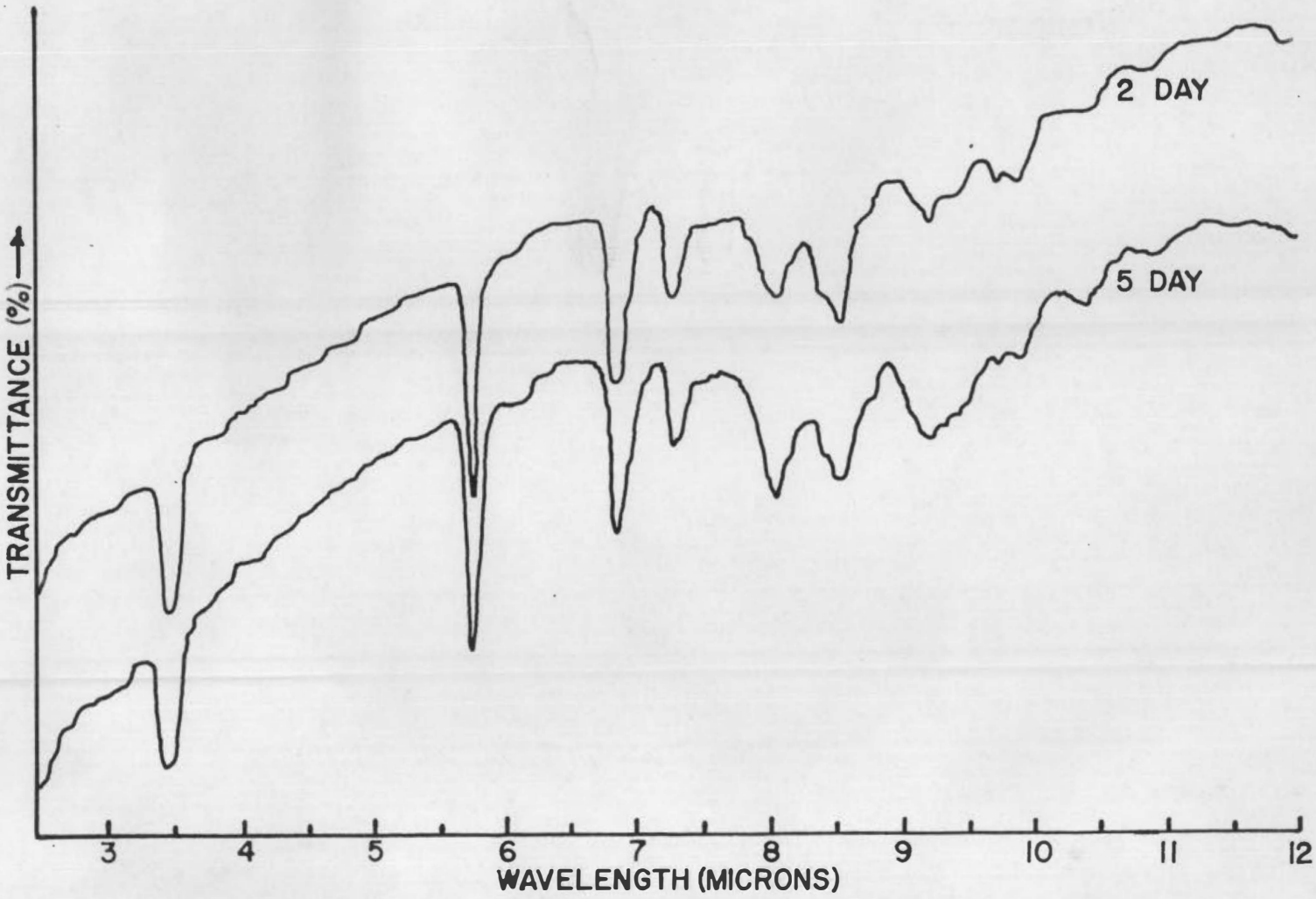

Figure 3. Infrared absorption spectra of lipid extracted from the livers of chicks at the ages of two and five days.

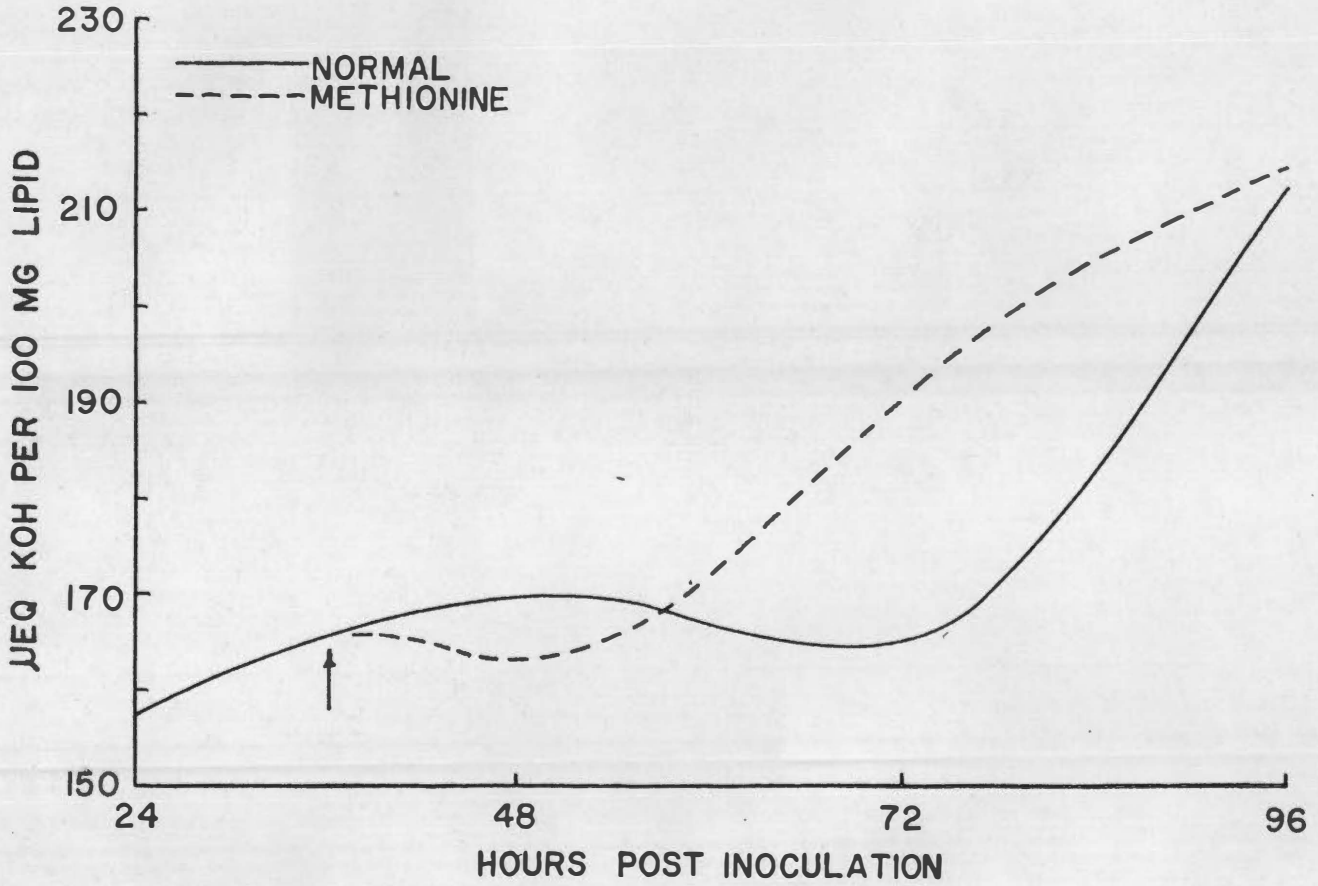

Figure 4. Saponification value of lipid extracted from the livers of normal chicks and the effect of administration of 50 mg methionine at thirty-six hours.

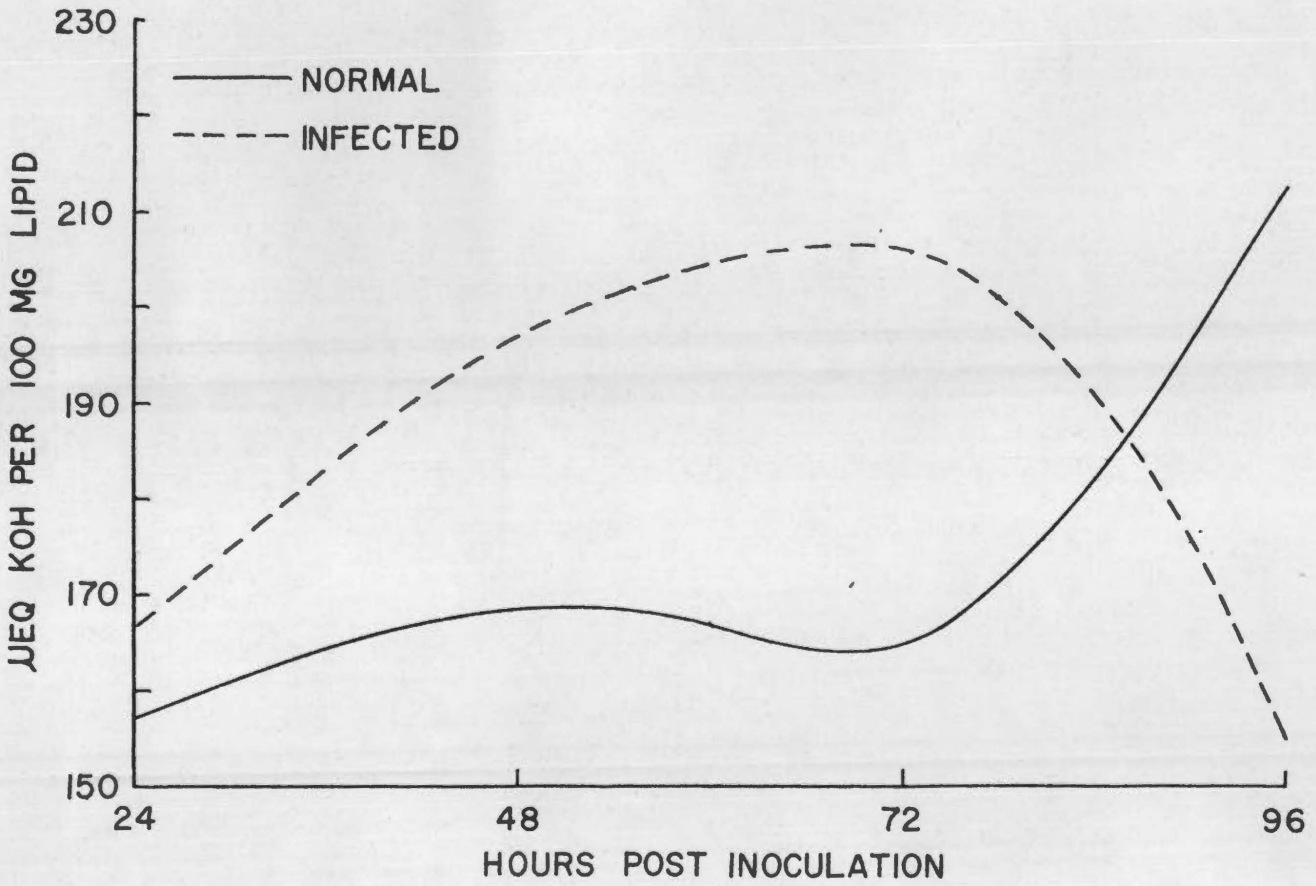

Figure 5. Saponification value of lipid extracted from the livers of chicks infected with Salmonella pullorum compared with that from the normal chick.

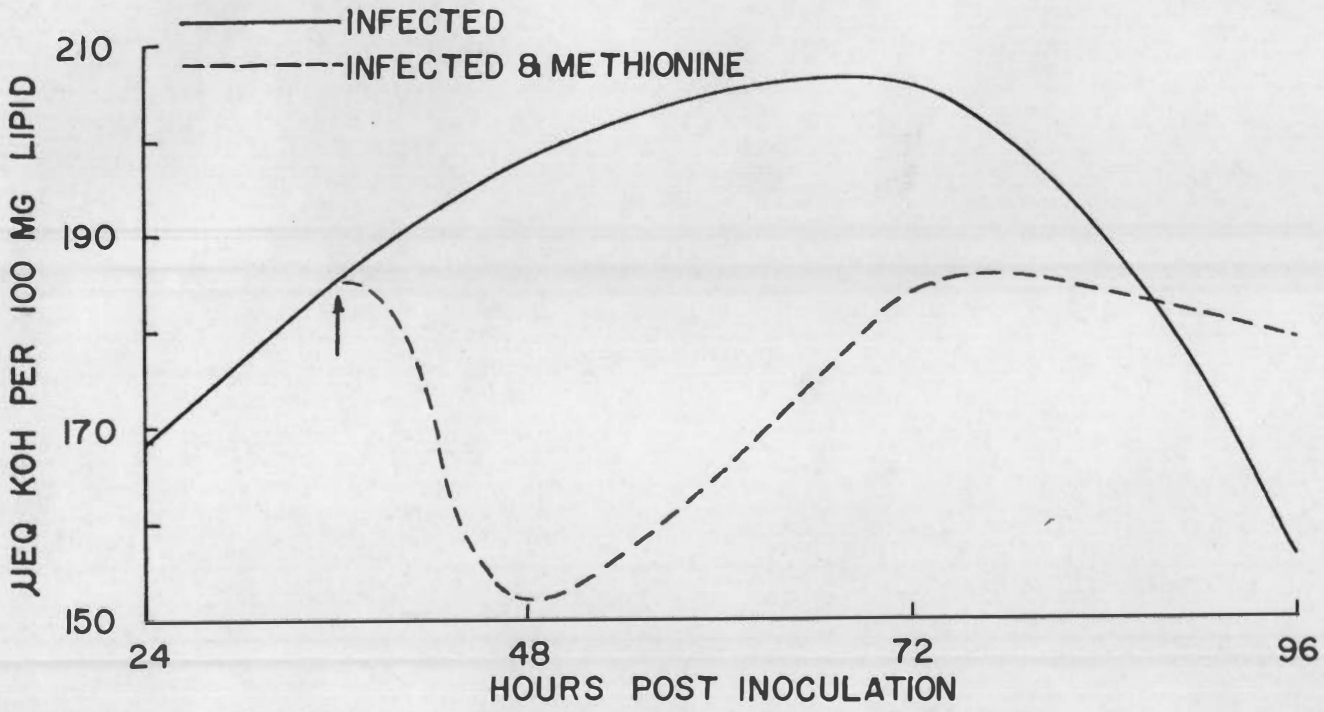

Figure 6. The effect of the administration of methionine on the saponifiable lipid in the chick infected with Salmonella pullorum.

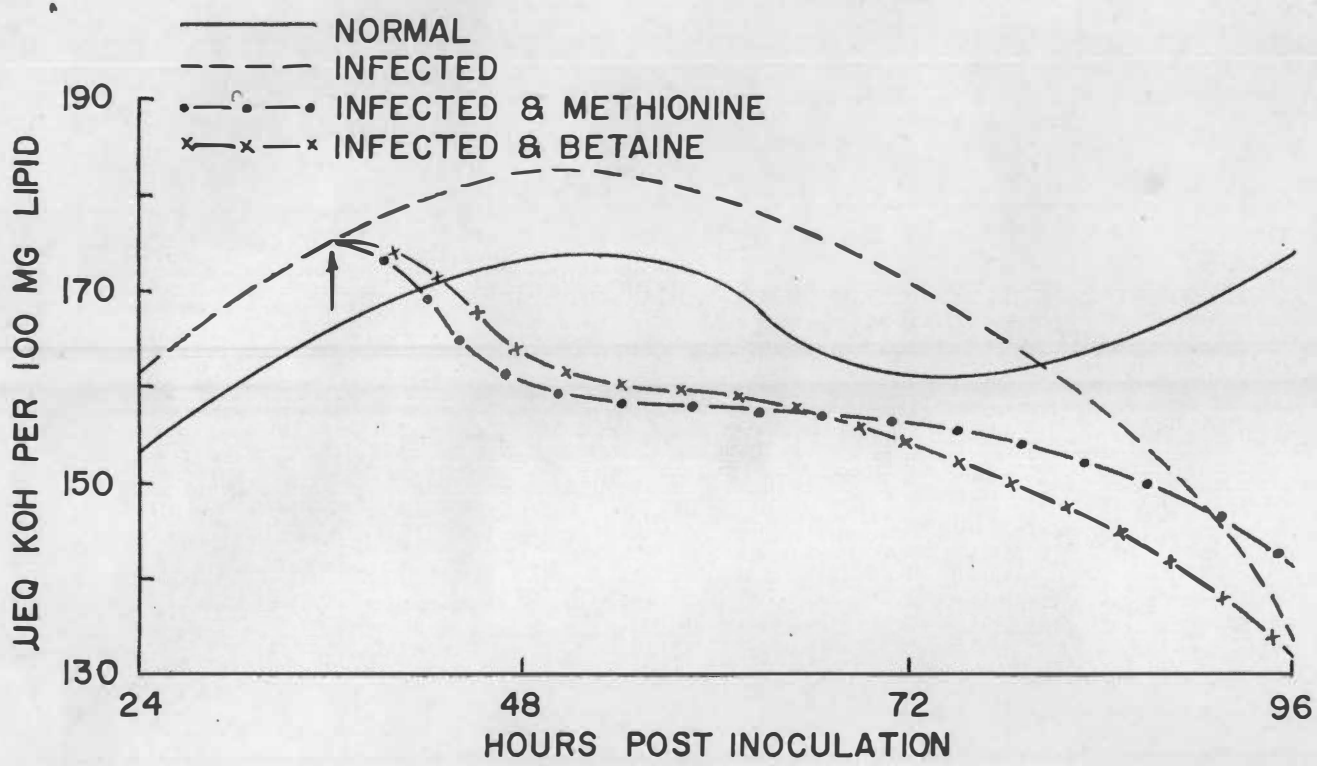

Figure 7. The effect of the administration of methionine and betaine on the saponifiable lipid of chicks infected with Salmonella pullorum.

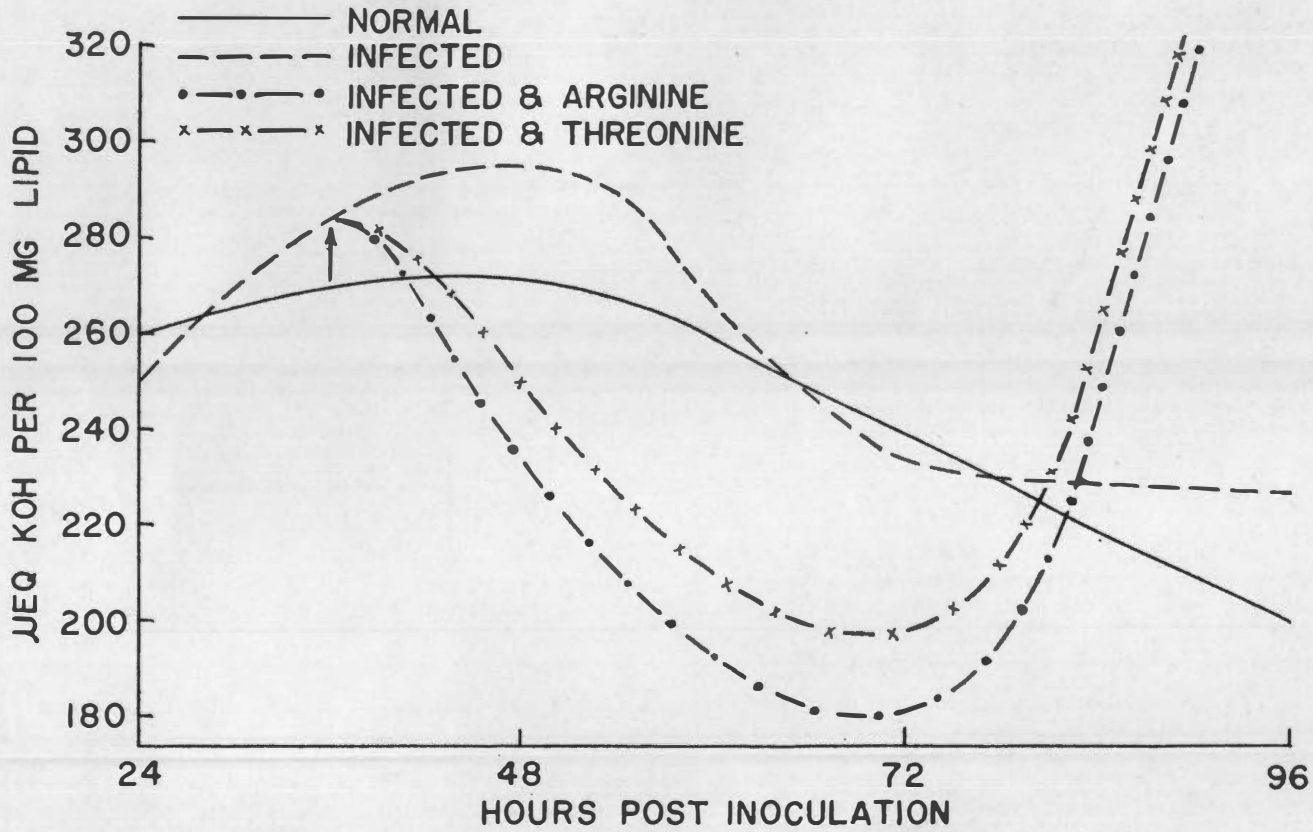

Figure 8. The effect of the administration of arginine and threonine on the saponifiable lipid of chicks infected with Salmonella pullorum.

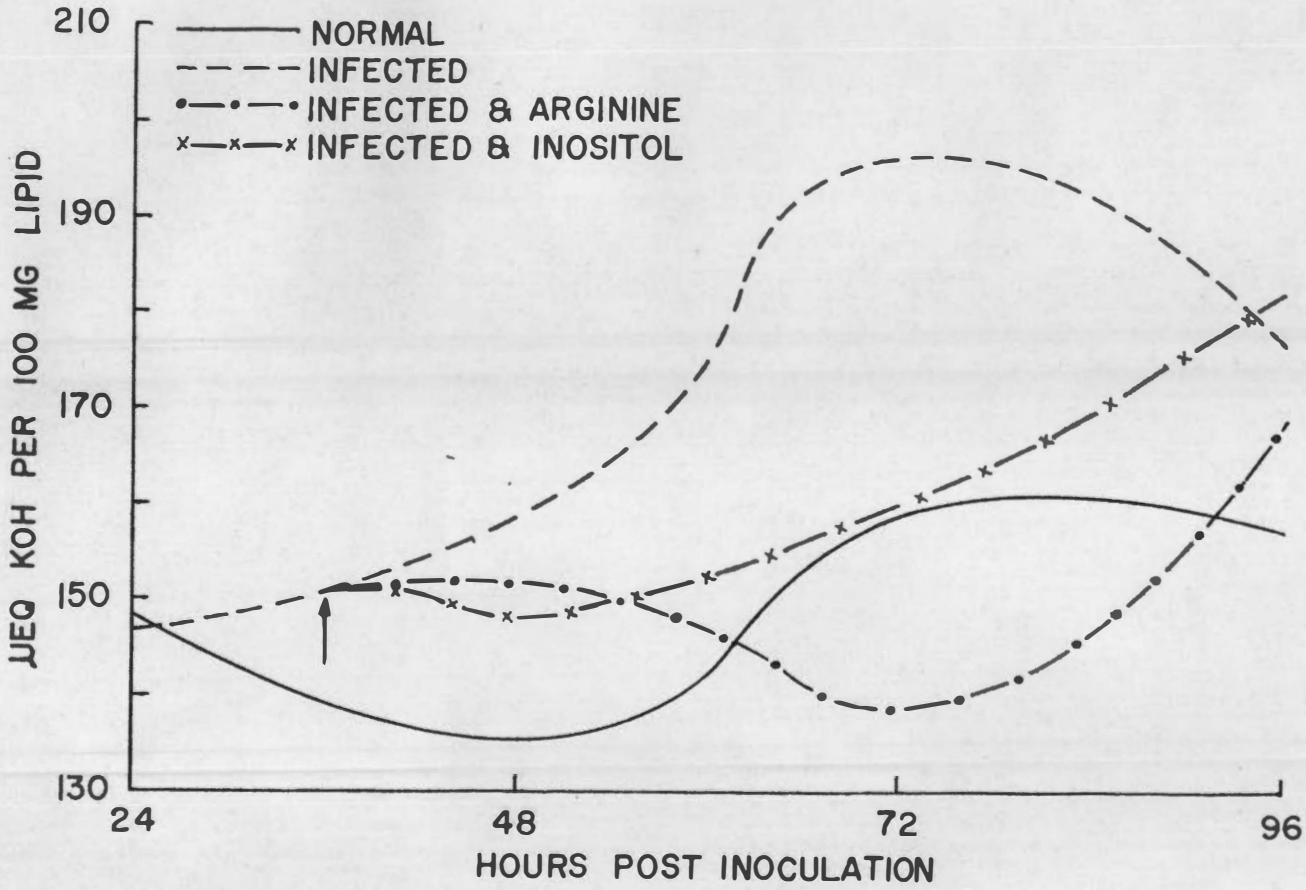

Figure 9. The effect of the administration of arginine and inositol on the saponifiable lipid of chicks infected with Salmonella pullorum.

TABLE 2

Activity (c/m) of P^{32} Labelled Phospholipid in 50 mg Liver Lipid of Chicks Four Hours Following Intraperitoneal Administration of 1 μc of $H_3P^{32}O_4$

Hours	Normal	Normal Methionine	Infected	Infected Methionine
24	522		386	
48	510	450	394	452
72	526	387	860	572
96	580	762	953	507

TABLE 3

The Activity (c/m) of P^{32} Labelled Phospholipid in the Liver of Chicks Four Hours Following Intraperitoneal Administration of 1 μc Labelled Inorganic Phosphate

Hour	Normal	Normal Arginine	Infected	Infected Arginine
24	452		668	
48	932	722	1019	744
72	733	914	860	833
96	836	787	720	905

VI. DISCUSSION

Lipid metabolism in the liver under conditions of stress has received a great deal of attention. This has been especially true of those conditions in which the liver is the primary site of the lesion such as poisoning with phosphorus, ethanol or halogenated organic compounds, or with living disease agents such as viral hepatitis and leptospirosis. There was for some time a question as to whether the infiltration of fat caused the lesion or whether the lesion gave rise to the fat. Apparently the metabolic changes which occur in the initial stages of injury subsequently give rise to fatty infiltration. Physiologic fatty infiltration of the liver has been ascribed to the dietary state of the individual, and it can be induced by feeding a diet high in fat and low in protein. In most of these cases the fat infiltration is rapidly alleviated by the addition of lipotropic agents to the diet.

It should be noted that the chick at the time of hatching may be considered to be on a high fat, low protein diet. Entenman, et al. (1940) reported chicks to have a fatty liver and hyperlipemia for about three to four weeks. This condition may be important in determining the high susceptibility of the chick to pullorum disease as resistance increases at about the time the lipids reach normal values, i.e., three to four weeks of age (Hamilton, 1932). It has been demonstrated that treatment to induce fatty liver, either dietary or toxic, increases the susceptibility of animals to pathogenic organisms (Formal, et al., 1958a, b).

An infection which would establish itself in an animal with a fatty liver and subsequently lead to a decrease in methionine, a lipotropic agent, would be expected to induce changes in the lipid composition. Such changes in chicks infected with Salmonella pullorum were found in the present investigation. These have been represented by a temporary increase in the saponification value of the lipid extracted from the livers of infected chicks (figures 4, 5, 6, 7, 8, 9). A change in the rate of incorporation of phosphate into phospholipid also has been found (tables 2, 3).

The infrared spectra of the gross lipid extracted from the liver of two to five day old chicks showed some changes (figure 3). The most significant changes were increased absorption at 8.1 μ and at 9.2 μ as the chick aged. These bands have been attributed to the organic phosphate of the phospholipid (Freeman, 1957). Spectra very similar to those of the normal were obtained from the lipid extracted from infected livers (figure 1). The spectra of the liver lipids from the infected chicks showed insufficient variation from those of the normal to indicate the type of change which might result. In view of the interference between cholesterol esters and triglycerides which results in overlapping of absorption bands (Freeman, 1957) little difference in the spectra would appear when the ratio of these compounds varied. Triglycerides and phospholipids give similar spectra due to their somewhat similar structure.

Though there was no detectable change in the infrared spectra, infection of the chick with S. pullorum did lead to a change in the saponifiable lipid. This change may be related to a shift in the ratio

of the various classes of lipids in the gross lipid extract which was obtained by the procedure used in this study. The percentage of lipid as triglyceride may increase or the chain length of the fatty acid radicals of the neutral fat may have been decreased. This then would account for the higher saponification value with a like weight of triglyceride and/or phospholipid. This alteration may also result from a number of other modifications such as the loss of the ability to synthesize cholesterol, cholesterol esters or the phosphatids. It has been demonstrated by a number of investigators (Christie and Judah, 1954; Hartmann, et al., 1953; Ludewig, et al., 1939; Tsuboi, et al., 1951; Winter, 1940) that following liver injury there is an increase in neutral fat. This increase in neutral fat was observed to be paralleled by an increase in the phospholipid. Winter (1940) has proposed that liver damage results in an alteration of fatty acid metabolism and thereby induces a fatty infiltration of the liver. These observations are in accord with the hypothesis set forth by Tsuboi, et al. (1951) in which the variation in lipid level was suggested to be due to a specific injury rather than to regeneration of the tissue following damage. On the other hand, Ludewig, et al. (1939) found neutral fat to increase in regenerating liver from partially hepatectomized rats. A peak was reached in two to three days and the lipid level returned to normal by about the seventh day. One finds, therefore, parallels between fatty changes due to other agents and those observed in this study.

Hendler (1958), however, has called attention to the importance of lipid in maintenance of the microsomal activity in protein synthesis.

He was able to show a decrease in the incorporation of phenylalanine -3-^{14}C into hen oviduct mince following treatment with lecithinase A, lysolecithin or deoxycholic acid. Treatment with ribonuclease resulted in far less disruption of the system than with the lipolytic agents. This would indicate an active relationship of the lipid with protein synthesis. The class of lipid exerting this effect was not identified, but was probably due to the phospholipid.

The studies concerned with phospholipid which were carried out in this research were of an insufficient scope to reflect on this hypothesis.

A number of agents aid in alleviating the fatty infiltration of the liver. Among the most important are those with labile methyl groups, choline, methionine, lipocaic, etc. However, there have been reports which indicate other amino acids to be effective, but usually these are subject to controversy.

Of the lipotropic agents choline has had the widest use (Best 1950, 1956), but this substance was found to be highly toxic to chicks upon parenteral administration. Thus, it did not lend itself to the experimental plan in this study.

In these experiments the reduction of the saponifiable lipid fraction and alteration of the rate of incorporation of labelled phosphate into the phospholipid of the chick liver demonstrated the lipotropic action of methionine. This effect of methionine may be attributed not to the involvement of this amino acid in synthesis of choline and subsequently phospholipid, but to its far more important role in protein synthesis or alleviation of toxic effects on other

organs of the body (Drill, 1952).

Threonine caused a reduction in saponifiable lipid in the liver of the chick infected with S. pullorum, the effect being comparable with that achieved by methionine and arginine. Threonine, an essential amino acid, has been demonstrated to exert an effect upon fat metabolism. This activity depends upon the presence of small amounts of choline (Singal, et al., 1953a, b). It was suggested that threonine exerts an effect quite different from that of choline. Possibly it is required for synthesis of phospholipid synthesizing enzymes, or it may be involved in a similar way in the metabolism of the nucleic acids.

Arginine markedly decreased the amount of saponifiable lipid in the liver of chicks infected with S. pullorum. Its effect was notable in its duration as well as its degree in comparison to inositol. The effect of this amino acid on the incorporation of phosphate into phospholipid in the liver of infected chicks was generally to reduce the activity below that of the infected control. Such activity has not previously been reported for this amino acid.

Betaine gave evidence of being somewhat toxic for the chick in comparison with the amino acids. As a result of this finding the dose level was reduced to one-fifth that of the amino acids, i.e. to 10 mg per chick. This dose led to a notable mortality rate among normal controls as well as apparently exerting no particularly beneficial results on the infected bird. There was, however, a reduction in the saponifiable lipid fraction of the liver lipid. This may have been due to synthesis of choline or to the glycine which results upon demethylation of betaine. On the other hand, an important role may be the

methylation of homocysteine, as betaine has been demonstrated to be the immediate donor of methyl groups in this reaction (Muntz, 1950; Artom and Crowder, 1950). This latter activity may explain the similar effects of betaine and methionine and especially the apparently more extended effect of betaine.

Inositol has been found to participate in the synthesis of the phospholipid. Its effect on clearance of saponifiable lipid from the liver of the infected chick was markedly less pronounced than treatment with arginine; however, in the first sample taken following the administration of the therapeutic agents inositol exerted a greater effect. Hartmann, et al. (1953) found that methionine and inositol exerted similar effects. When animals fed a protein deficient diet were placed on the same diet supplemented with inositol a marked increase in the excretion of amino acids, in both the urine and feces, resulted (Hartmann, 1954). It was suggested that this was due to inositol activating a system or removing an inhibitory system which then permitted autolysis of protein. Methionine would be liberated in this reaction and be free to exert its well known lipotropic effect.

The findings in the present study further indicate the importance of maintaining a metabolic pool of amino acids. Ross, et al. (1955a, b) and Dooley (1957) reported certain amino acids exerted a protective effect and offered hypotheses to explain this action. While these most certainly account for some of the protection afforded by these amino acids there may be others of equal or greater importance.

The failure of inositol to act to the same degree as the amino acids suggests the protective effects of the latter to have been due to

something other than their lipotropic action. DiLuzio and Zilversmit (1956) reported that induction of fatty liver by high-fat, low-protein diets was not related only to the deficiency of choline, but to the protein deficiency.

The results of the present study lead to the assumption that the changes in the lipid are incidental to numerous other changes in metabolism which occur in pullorum disease. It is suggested that one of the most important of these changes is to be found in an interference with protein metabolism. This may be a direct result of the loss of free amino acids from the metabolic pool as has been reported by Ross (1956) and Dooley (1957). Or, alternatively, it may be the result of a loss of the energy yielding mechanisms, a conjecture for which there is supporting data (Dooley, 1957).

The clinical symptoms of pullorum disease would indicate both the above effects to occur and to be complicated by anorexia. The nutritional state of the chick during the diseased condition is quite poor, and this is complicated by the tendency to the fatty state due to the absorption of yolk material. The general lipid changes observed, however, were probably due to the unbalancing of the nutritional state of the chick.

VII. SUMMARY

The effect of infection of chicks with Salmonella pullorum upon the lipid content of the liver has been investigated. The infrared spectra of the lipid from the normal and infected chicks showed no discernible differences. Some differences were observed to occur in the infrared absorption of the lipids as the chick aged. The saponification values of the lipids from the infected chick were found to increase above the values of the normals and then decline to a level below the original. The saponification values of the lipids from normal chicks showed a slight rise through the experimental period.

The rate at which labelled inorganic phosphate was incorporated into the lipid fraction was variable. In general the uptake was more rapid in the infected chick than the normal.

Therapy with amino acids and lipotropic agents was attempted with some success. Arginine, methionine and threonine each caused varying degrees of reduction in saponification values. Arginine and methionine were found to reduce the rate at which labelled inorganic phosphate was incorporated into the phospholipid of the infected chick liver.

The lipotropic agents, betaine and inositol, induced lowered saponification values for lipids from livers of chicks infected with S. pullorum. These agents were less effective than amino acids. The cause for this effect was discussed with relation to the effects of these agents on protein metabolism. Choline administered intraperitoneal was very toxic for chicks and therefore was not used in this research.

The relationship of the lipid changes with the disease has been discussed and suggested to be incidental to other metabolic changes which occur during pullorum disease. In view of the relative therapeutic effects of the amino acids and lipotropic agents, the amino acids and probably thereby the protein metabolism have been implicated as leading to the lipid changes observed in chicks infected with S. pullorum.

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