VITAMIN A DEFICIENCY AND *EIMERIA ACERVULINA* INFECTION IN THE CHICK

by

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ABSTRACT

The present investigation was conducted to determine the effect of an infection with Eimeria acervulina on vitamin A deficient birds. One hundred day-old chicks were divided into 2 equal groups, and one group was raised on 440 I.U. vitamin A per kilogram of feed, while the second group received the normally recommended 4400 I.U. per kilogram. At 5 weeks of age, half the birds from each group were given an immunizing infection with 3 million oocysts of E. acervulina. The clinical symptoms were more acute, and the oocyst production, mortality, and weight losses significantly higher, in the birds on the low vitamin A diet.

When the birds were 9 weeks old, half were subjected to a second infection and the other half held until they were 19 weeks old. The 9-week-old birds were given an oral inoculation of 10 million oocysts to evaluate the effect of a low vitamin A diet on: the strength of the immune response; a primary infection with E. acervulina in 9-week-old birds; and the early tissue stages of the parasite in immune and non-immune birds. The results from this experiment showed that the degree of immunity is not affected by a low vitamin A ration, but primarily determined by the severity of the initial infection; and that the primary infection in 9-week-old chicks is clinically very similar to that in younger birds. The histological examinations

demonstrated that the low vitamin A level used in this study did not affect the integrity of the intestinal epithelium, nor the development of the endogenous forms of the parasite. It was also shown that the sporozoite invasion was the same in immune and non-immune birds, but the schizont development was severely inhibited in the immune birds regardless of the vitamin A level. The non-immune birds on the low vitamin A ration did not show as distinct a heterophil response to infection as did the other groups.

The remaining birds, half which were immunized, were maintained on their respective diets until 19 weeks of age, and during this period there was no clinical evidence of a vitamin A deficiency, and the growth rate was not significantly affected by the level of vitamin A in the diet. When 19 weeks old, the birds were inoculated with 25 million oocysts of E. acervulina to assess the effect of an extremely heavy primary infection in older birds, and to evaluate the degree of immunity still retained 14 weeks after an initial infection. The mortality was very high, and the weight losses significantly greater, in birds on the low vitamin A dietary level. Immunity, although decreased from the earlier experiment, was still present in birds on both levels of vitamin A.

It was concluded from these experiments that a suboptimal level of vitamin A in the diet increased the pathogenicity of E. acervulina in chickens of various ages. The low vitamin A level did not, however, significantly affect the bird's immune response.

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I. INTRODUCTION

Experimental evidence has demonstrated that Eimeria acervulina is not a very pathogenic species of coccidia. However, it has been noted that under field conditions an infection with this parasite is often associated with severe weight losses, morbidity, and some degree of mortality. Therefore, it may be assumed that the pathogenicity of E. acervulina is increased by certain external factors such as environmental conditions and diet. As it has been demonstrated that low levels of vitamin A in the ration will increase the degree of parasitism in all animals, it is suggested that the pathogenicity of E. acervulina may be increased by a deficiency of vitamin A. This thesis was examined in the following investigation which compared the effects of E. acervulina infections in birds raised on 2 levels of vitamin A in the diet. The pathogenicity was assessed by a comparison of oocyst production, weight gains, and mortality. Histological examination was used to determine if any pathology of the intestinal epithelium resulted from the low vitamin A diet. The response of the immune host to a coccidial infection was also investigated in birds on both dietary vitamin A levels.

II. REVIEW OF LITERATURE

A. Vitamin A and Coccidiosis

The relationship between vitamin A and parasitism in chickens was first investigated over 40 years ago by Ackert and coworkers (1927). Experimenting with the chicken roundworm, Ascardia lineata, Ackert found that this parasite became better established in birds on a low vitamin A diet than in birds receiving a normal ration. Further work by this author (1931) (1942) verified the earlier findings, and showed that birds on a vitamin A deficient diet had a much slower peristalsis than birds receiving normal levels of the vitamin. He suggested that this fact might account for the establishment of more and larger worms in the low vitamin A group. Murphy et al (1938) reported a correlation between caecal coccidiosis and diet. During the course of an experiment, to assess the effects of varying amounts of cod liver oil in a complete diet, the birds contacted caecal coccidiosis. It was noted that the birds on the lower levels of oil showed higher mortality, and took longer to regain their weights, than the birds getting 1% oil in their ration. Taylor and Russell (1946) established, through a series of experiments, that the minimum requirements for vitamin A recommended at that time were not adequate under

stress conditions. Although 1200 I.U. of provitamin A per pound of diet were adequate for normal growth, birds subjected to caecal coccidiosis were far more affected by the disease than birds fed higher levels. They recommended, therefore, that a 66% safety margin be added to the standard vitamin A level, to give 2000 I.U./lb. feed to provide protection in time of stress. This increased need for vitamin A in a coccidial infection is allied to a drop in vitamin A liver reserves. Davies (1952) found that the normal liver reserve of vitamin A in a chick was about 292 I.U./gram, and that this dropped to an average of 15 I.U./gram during a mixed infection with Eimeria tenella and E. necatrix. In these experiments the birds were receiving 2100 micrograms of carotene in the feed, equivalent to 3500 I.U. vitamin A per pound of feed. Davis attributed the liver loss to the lack of conversion of carotene to vitamin A, as this changeover occurs in the epithelium of the intestine. Erasmus, Levine, and Scott (1958) (1960) believed that the reported drop in liver reserves of vitamin A could be due to a lowered feed intake during a coccidiosis infection. Therefore, in their investigations on the interrelationship between coccidiosis and vitamin A, the controls were restricted to the same feed intake as the infected birds. Employing this refinement of technique, and using a mixed culture of E. tenella and E. acervulina as

inoculum, they conducted experiments with low and high levels of vitamin A. The following conclusions were reached:

1) Following a coccidiosis infection, the birds on the high levels of vitamin A showed a better appetite, and faster growth rate, than the birds on low levels of dietary vitamin A.
2) The chick's requirement for vitamin A is increased by coccidiosis. This was confirmed by greatly reduced liver storage of vitamin A in all infected groups regardless of the level in the diet, and when controls were restricted to the same feed intake.
3) Carotene as a source of vitamin A is not as effective as the preformed vitamin.

The authors did not obtain consistent results on the correlation between vitamin A levels and the severity of the disease. However, Garriets (1961) found a direct relationship between the level of vitamin A in the diet and the severity of caecal coccidiosis. In fact he considered the degree of protection so convincing, he recommended that high levels (5000 I.U./kilo.) of vitamin A could be used prophylactically. However, this author relied on natural infection from contaminated litter, so his results are open to question. In a more controlled experiment, Waldroup et al (1963) concluded

that mortality was not affected by the level of vitamin A in the diet, and found there was no histopathological differences in the caecal epithelium between birds on low and normal vitamin A diets. However they did report a significant difference in the weight gains, the higher vitamin A groups regaining their lost weight much more rapidly than the birds on lower vitamin A levels. Panda and Combs (1964) working on liver vitamin A values, reported that when excessive amounts of vitamin A were fed (up to 24,000 I.U./kilo.) the proportionate amount of vitamin A retained in the liver at the end of a mixed intestinal coccidial infection was higher than found with normal dietary intakes.

B. Physiological Role of Vitamin A

The reason for this correlation between vitamin A and the severity of coccidiosis infection must depend on the role of vitamin A in the body. It has long been established that vitamin A is necessary for normal functioning of the epithelium. As early as 1925 Wolbach and Howe studied the tissue changes resulting from avitaminosis A in rats. They reported that a squamous, stratified, keratinizing epithelium slowly replaced the normal columnar epithelium of the mucous membranes, although the goblet cells themselves were not affected. They found that the respiratory mucosa was first affected, followed by the

epithelium of the salivary glands, reproductive, and urogenital tract. There appeared to be no change in the epithelium of the stomach and intestines. However at a later date, Richards (1935) reported that the earliest microscopic indication of avitaminosis A in the rat was keratinization in the digestive tract, which showed up clinically as a gastro-enteritis. Seifried (1930) studied avitaminosis A in chickens and found the typical keratinization of mucosa as reported for the rat. He reported that the histopathology of avitaminosis A in the chick was primarily in the respiratory tract, wherein the normal tissue was replaced by squamous keratinizing epithelium. This resulted in a loss of such specialized structures as ciliated cells and goblet cells, and consequently in a loss of the protective properties of the lining epithelium. In the alimentary tract the changes were confined to the mucous glands and ducts of the upper regions. There were no experiments conducted on the intestinal epithelium. Elvehjem and Neu (1932), in studies on avitaminosis A in chicks, found one of the most consistent findings was an elevated uric acid content in the blood. This level was dependent on the degree of kidney damage from keratinization of the tubules, preventing the normal elimination of uric acid from the body. The authors also commented on the characteristic ataxia associated with avitaminosis A and attributed it to

nerve damage rather than to uremia. However, later studies by Wollam and Millen (1955) have shown that an increase in cerebrospinal fluid is the main cause of this disorder. Roels (1967), in a review of vitamin A functions, has suggested that the general role of this vitamin in metabolism is that of a "membrane active" compound, concerned with the stability of cell membranes. It is also implicated in protein and lipid metabolism and the stability of lysosomes. A very consistent finding in cases of vitamin A deficiency is a depressed glycogenesis resulting from impaired adrenal gland function. The influence of vitamin A on the adrenocortex of chicks was investigated by Glick (1963). He reported that 5-week-old birds on a low vitamin A diet showed reduced adrenal cortical function as reflected by a significantly lower heterophil increase after ACTH injection.

The relationship between avitaminosis A and immunity to disease has been investigated by many workers. In 1960, Harmon et al demonstrated the effect of a vitamin A deficiency on the antibody-producing ability of swine. They reported that pigs on a low vitamin A diet produced lower antibody titres against Salmonella pullorum than pigs on a normal diet. Yaeger and Miller (1963) experimented with the susceptibility of rats to Trypanosoma cruzi under conditions of avitaminosis A. They found that rats on vitamin A deficient diets showed less

resistance to infection with this parasite, and also to miscellaneous bacterial infections, than animals receiving adequate amounts of the vitamin. Panda and Combs (1963) working with S. pullorum infection in chicks, investigated the effects of suboptimal levels of vitamin A in the ration. They fed diets containing 9960 I.U. vitamin A per kilogram of feed, and 975 I.U./kilogram, and were able to demonstrate a significantly lower agglutination response to S. pullorum, and also a lower bursa weight, in birds on the lower levels of vitamin A. This implication of the bursa of Fabricius in antibody response confirms earlier work by Glick, Chang, and Jaap (1956). They reported that in their investigation, only 8/75 bursectomized birds produced antibodies against S. typhimurium, compared to 63/73 of the controls. Challey (1962), working with Eimeria tenella infections in normal and bursectomized birds, observed a much higher mortality in the bursectomized chicks. However, these findings were not confirmed in later investigations by Pierce and Long (1965), and Rose (1968).

C. Eimeria acervulina Tyzzer 1929

E. acervulina is the species of Eimeria most commonly reported from diagnostic laboratories, and also the least well controlled by coccidiostats. It affects the upper half of the small intestine, particularly the duodenum, usually causing

whitish transverse streaks on the mucosa. This species was first described by Tyzzer (1929), after isolation from intestinal lesions. Acervulina is a Latin term which is descriptive of the species' intestinal pathology: acerv = a heap and lina = line. Infections with this species are characterized by chronic, rather than acute, symptoms which include anorexia, diarrhea, and reduced weight gains. E. acervulina infections may be differenciated from infection with other species of Eimeria by the prepatent period of 4 days; large numbers of oocysts passed; and the relative lack of pathogenicity.

1. Life Cycle

The life cycle begins with the oocyst passed out in the feces. Tyzzer (1929) reported the oocyst as egg-shaped and having an average measurement of 19.5 by 14.3 microns, with a range of 17.7-20.2 microns in length, and 13.7-16.3 microns in width. Becker (1956) disagreed with these measurements, finding the oocyst to be smaller on the average, and reported the average size as 16.4 by 13 microns. Lund and Farr (1965) agree with these measurements, but other authors have listed different average sizes: 17 by 14 microns (Morehouse and McGuire, 1958); 18.3 by 14.6 microns (Edgar, 1955); and 17.4 by 13 microns (Long, 1967). For this investigation the average size of the oocysts were 17.3 by 15 microns. This great variation in

reported sizes demonstrates the difficulty in making a species diagnosis from the oocyst size alone.

As with all species of Eimeria, the newly passed oocyst is unsporulated and consists of a dense, tough, outer covering surrounding a sporont or zygote. Oxygen, moisture, and heat are required for sporulation which begins with the sporont undergoing reduction division and throwing off a polar body. The sporont then divides into 4 sporoblasts, each of which develops into a sporocyst. Two sporozoites develop in each sporocyst, giving a total of 8 sporozoites per oocyst. This arrangement of 2 sporozoites within 4 sporocysts is characteristic of the genus Eimeria, and separates it from the genus Isospora, in which the oocyst contains 2 sporocysts, each having 4 sporozoites. Wilson and Fairbairn (1961), studying the sporulation of E. acervulina when incubated at 30°C, demonstrated maximum sporoblast formation at 10 hours, and maximum sporozoite development at 20 hours. Edgar (1955) reported sporulation as early as 17 hours at 29°, and Krasser (1963) was able to initiate a slight infection in birds with oocysts showing sporulation at 13 hours. However all authors agree that maximum sporulation occurs between 24 and 27 hours at 28°-30°C. The survival of Eimeria oocysts outside the body depends primarily on moisture as demonstrated by Davies and Joyner

(1955). Farr and Wehr (1949) in a study of oocyst survival in the soil, concluded that humidity is the limiting factor, and for normal viability of the oocyst it should exceed 90%. They were able to demonstrate the survival of E. acervulina oocysts for over 86 weeks under moist shady conditions.

Under normal conditions a bird must ingest the sporulated oocyst to become infected, and infection begins with the fracture of the oocyst wall, excystation, and release of sporozoites in the intestine. Doran and Farr (1962; 1965), Doran (1966), and Farr and Doran (1962), have reported their investigations into the factors involved in the initial sporozoite release. According to these authors, the following steps are involved:

1) The wall of the oocyst is fractured through mechanical action of the gizzard and the sporocysts are released.
2) Through the action of pancreatic juice, in the presence of bile, there is activation of the sporozoite and a probable digestion of the sporocyst plug which allows the sporozoites to escape.

From in vitro experiments it would appear that the critical enzymes in the pancreatic juice are trypsin and chymotrypsin, and it is interesting to note that Britton et al (1964) have found a marked decrease in excystation of oocysts in birds on a low protein diet which produces low trypsin values.

Nyberg and co-workers (1968) have suggested that carbon dioxide is the initial stimuli for the excystation of E. tenella oocysts, and Lotze and Leek (1968) have demonstrated that some sporozoites, after activation with carbon dioxide and enzymes, can escape through the micropyle of the sporocyst and emerge from an undamaged oocyst through an unseen hole. It is apparent that not all the factors necessary for excystation have been elucidated. Experiments by Landers (1960) and Davies and Joyner (1962) have shown that intestinal infections with Eimeria species can be initiated through parenteral injections of sporulated oocysts. Doran (1966 a, b, c) has demonstrated that E. acervulina sporozoites can excyst and penetrate the villar epithelium as early as 10 minutes after ingestion of sporulated oocysts. He found that the time of greatest penetration was 1-2 hours after dosing, and that the area of greatest invasion was at a point just before the curvature of the duodenal loop, to about 2.5 cm. before the entry of the pancreatic duct into the ascending duodenum. The endogenous stages of E. acervulina are confined to the epithelium of the small intestine as described first by Tyzzer (1929) and confirmed by Doran (1966, a, b, c), Vetterling and Doran (1966), and Pout (1967). Working with E. necatrix, Van Doorninck and Becker (1957) first demonstrated that the sporozoites were engulfed by macrophages

as they entered the epithelium of the villi, and were transported down through the lamina propria to the epithelial cells of crypts before initiating schizogony. Challey and Burns (1959) reported the same migration of sporozoites in E. tenella, infections, and Doran (1966c) in E. acervulina infections. Doran found that sporozoites are not seen in the glandular epithelium until 18 hours after ingestion of sporulated oocysts, so this finding would preclude the possibility of direct penetration. He described the sporozoite as being pointed anteriorly and averaging 8 microns in length and 1.6 microns in width when free in the lumen, and about 4 microns in length when in the tissue. It is characterised by a eosinophilic refractile globule.

The schizogonous cycle consists of 3 schizont stages according to Warren and Ball (1967) and Long (1967). However, Vetterling and Doran (1966) who have done an exhaustive study into the endogenous forms of E. acervulina, report the presence of a 4th schizont stage. They demonstrated that the schizogonous cycle starts with the appearance of the sporozoite in the epithelial cell at the base of the crypt. Here the sporozoite rounds up, distal to the nucleus and just below the brush border of the cell, and begins the trophozoite or growth phase. When it reaches a certain size nuclear division occurs and the first

generation schizont is formed. This schizont is mature from 36-48 hours after ingestion of the oocyst, measures 10 by 7 microns, and contains from 8-16 merozoites. However, Long (1967) reports the size as 5-8 microns. When the merozoites are released they invade cells in the neck of the crypt and within 8 hours develop into second generation schizonts, measuring 5 by 4 microns, and containing 16 merozoites on the average. These merozoites, when released, penetrate the cells at the base of the villi. The third generation schizont that matures 16 hours later, or 56-72 hours after the initial inoculation of oocysts, is similar in size to the 2nd generation schizont but divides into only 8 merozoites on the average. These 3rd generation merozoites invade the epithelial cells of the villi, mostly in the middle region, and develop into the fourth generation schizonts which are larger than the previous 2 generations, measuring about 8 microns and containing 28-36 large merozoites. The gametogenous stages are initiated by the 3rd or 4th generation merozoites and are usually found in the tips of the villi. The microgametocytes which appear first at around 84 hours after oocyst inoculation, measure about 7 microns in diameter, and when mature contain about 90 micro-gametes. These average 3 by 0.5 microns and have 2 long flagella arising from the anterior end. The macrogametes are

seen first at 88-90 hours, and when fertilized by the released microgametes develop into oocysts. The oocysts are voided from the body 96-97 hours after inoculation of the infecting dose, and this short prepatent period is characteristic of infection with E. acervulina.

There are varying reports on the times, sizes, and locations of the different generations of schizonts. Tyzzer (1929) could not differentiate between the schizogonous stages and made reference only to the fact that schizonts were found near the tips of the villi, which would probably refer to the 3rd or 4th generation. The fact that there is variation in the times of maturation for the individual schizonts makes it difficult to accurately distinguish the exact stage encountered. However, all the investigators are in agreement that there are stages in the endogenous development progressing from the base of the crypt to the tip of the accompanying villi. Pout (1967) reported that the endogenous phases of the parasites seem to "ride" in the migrating host cells so that "trains" of parasites can be seen proceeding up the villus. He found this most marked at day 4 when maturing oocysts were seen at the tips of the villi with later generations of schizonts travelling behind them lower down in the villi. Vetterling and Doran (1966) reported that all the stages from sporozoite to gamete occur within the cells

in the gland invaded by the sporozoite and in the villi adjacent to it. They speculate that this movement further toward the lumen with each successive generation is more likely due to the flow of mucous than to epithelial cell migration. This concentration of the endogenous forms in limited areas accounts for the white-grey raised streaks which characterize a light infection with E. acervulina.

2. Pathogenicity

E. acervulina has never been considered a very pathogenic species of Eimeria. Tyzzer (1929) (1932) reported that the pathogenicity of the organism depended on the duration, as well as the intensity of infection. He stated that extensive involvement of the epithelium in severe cases would seriously interfere with digestion and absorption. Dickinson and Scofield (1939) investigated the clinical manifestations of large doses of E. acervulina, (35-50 million oocysts/bird) and confirmed Tyzzer's contention that mortality was not a factor. They demonstrated a decrease in food consumption and body weight from 4-10 days after inoculation, and reported that the return to pre-inoculation weight took 18-24 days. Dickinson (1941) investigated the effects of bird age and oocysts dose on pathogenicity. He concluded that a daily ingestion of 5000 oocysts was the margin between clinical and subclinical infections. He

concurred with earlier work that there was no mortality, the losses in body weight were temporary, and that if the infective dose was large enough there were clinical symptoms on day 4-9. These symptoms were anorexia, depression, loss of weight, and scanty, loose droppings. Morehouse and McGuire (1956) (1958) disagreed with the assumption that E. acervulina could not cause mortality. They demonstrated that successive inoculations with large numbers of oocysts could result in 75% mortality in some groups. They also reported that weight losses could reach 20% with a single heavy infection. These authors also demonstrated that the typical intestinal lesions of an E. acervulina infection were present only in very light infections of under 500,000 oocysts. Heavier infective doses of 5 million or more oocysts/ bird resulted in a marked inflammation and thickening of the intestinal wall. Hein (1968a) recently investigated the effects of variable doses of E. acervulina oocysts on young chicks. She found that the size of dose was directly correlated with the severity of the lesions and the degree of weight loss. Anorexia and morbidity were seen only at the higher levels of infection - 5 million and 20 million oocysts/bird. There was no mortality in any groups, but pathogenicity of the parasite was higher in the younger birds. The large numbers of oocysts needed to give clinical evidence of disease is related to the findings of all

investigators that a high oocyst production is typical of infection with E. acervulina. Morehouse and McGuire (1958) reported that one infective dose of 5 million oocysts resulted in a total elimination of 684 million oocysts per bird, and Long (1967) has demonstrated 2000 million per bird from a dose of 100,000 sporulated oocysts.

3. Histopathology

The pathological findings in a bird infected with E. acervulina depend on the number of sporulated oocysts ingested at one time. The previously mentioned white patches or streaks resulting from agglomerations of schizonts or oocysts, appear in light infections and are found mainly in the duodenal loop. As the infection increases there is a more extensive involvement of the same fundamental type accompanied by an edematous swelling of the epithelial layer and lamina propria causing a thickening of the intestinal wall. Hein (1968a) reported that, with heavy infections, the lesions extended further down the intestine. An extensive vasodilation occurs in heavy infections resulting in inflammation in the invaded areas of the intestine. Sloughing of the intestinal epithelium is present in both light and heavy infections according to Morehouse and McGuire (1958). Pout (1967) has shown that on day 4 there is a significant decrease in the height of the villi to the total mucosal

thickness in E. acervulina infections.

D. Immunity to Coccidiosis

Tyzzer (1929) investigated immunity in coccidial infections and concluded that the most pronounced protection occurred in species that penetrate deeply and tend to be retained in host tissues - E. tenella for example. He reported poor immunity in species such as E. acervulina that develop superficially in the epithelium. Peterson (1949) reported that repeated exposures to E. acervulina are necessary for a good degree of immunity. He found that 3 light inoculations of oocysts (10-50 thousand) in 2-week-old chicks gave complete clinical protection when challenged 3 weeks later. However, although no lesions were present, a few oocysts were still passed indicating that complete immunity was not achieved. This confirms earlier work by Dickinson (1941) who reported the presence of oocysts in the droppings of clinically immune birds. However he did find that a single large inoculum of oocysts was as effective as several small doses in confering this immunity. He demonstrated that retention of protective immunity at 70 days, was identical either following a single dose of 25 million oocysts/bird of E. acervulina or multiple small doses totalling this amount. Rose and Long (1962) investigating complete immunity with Eimeria species, infected birds with 500,000, 5 million, and 10 million

oocysts of E. acervulina on 3 consecutive weeks. When challenged 18 days later with 20 million oocysts, the birds were completely immune to the parasite as verified by negative fecal oocyst counts. Hein (1968b) produced effective protective immunity with 2 small immunizing doses 2 weeks apart. The inoculum consisted of 80,000 and 160,000 oocysts of E. acervulina, and the birds were challenged with 5 million oocysts at 28 days after the second inoculation.

Becker (1935) was one of the first to study the nature of coccidial immunity. He experimented with E. miyairii (syn. E. nieschulzi) in rats and concluded that there was no generalized host response. Unable to demonstrate protective humoral antibodies, he speculated that infection resulted in a localized response, and the nature of this was possibly a "sensitisation" of the epithelium which blocked the entrance of sporozoites. Morehouse (1938) agreed with this hypothesis, and in further studies with E. miyairii reported that significantly fewer sporozoites invaded the immune epithelium. However, various investigations into coccidial immunity in chickens have not supported these findings. Both Tyzzer (1932), in studies on E. necatrix immunity, and Horton-Smith and co-workers (1963) examining the immune response to E. tenella, found no difference in sporozoite invasion in immune or susceptible birds. It was

found that the immune mechanism inhibited the sporozoites from developing into schizonts, as no evidence of first or second generation schizonts were observed.

The presence of some type of protective humoral antibodies was demonstrated by Burns and Challey (1959) and Horton-Smith, Beattie, and Long (1961). In separate studies these investigators both used the same technique of ligating one caecum and then introducing an infection of E. tenella into the other. After an appropriate period of time, the isolated caecum was then exposed to infection and was found to be resistant. Pierce and co-workers (1962) reported the presence of precipitating antibodies in birds made immune to E. tenella. However the birds degree of resistance to further infection did not coincide with the level of circulating antibodies. Rose (1963) examined the nature of this humoral antibody. Using E. stiedae, and several chicken species of coccidia, she was able to stimulate the production of serum antibodies using antigens composed of intact oocysts; crushed oocysts; and tissue stages of the parasites. The antibodies were demonstrated by agar precipitation and complement fixation tests. Rose and Long (1962) were not able to correlate the presence of antibodies with resistance to infection, although the antibodies do exhibit destructive abilities as evidenced by their capacity to lyse

both sporozoites and merozoites *in vitro*. Rose (1963) has demonstrated lysing of sporozoites *in vivo* following intravenous injection in birds showing a high level of antibody. However, there is no apparent destruction following oral dosing. It has been conclusively demonstrated by many authors that there is no "booster" effect on the level of circulating antibodies when the bird is challenged with succeeding infections of coccidiosis. It has also been reported that the sera of resistant birds does not always contain antibody. Horton-Smith and co-workers (1963) studied the effects of immunity on the endogenous stages of the parasite and concluded that both schizogonous and gametogenous stages are inhibited. Horton-Smith (1963) has reported that the second schizogony is the stage of the life cycle which promotes the immune response under natural infections. This finding was confirmed by Rose (1967b) working with *E. tenella* and *E. necatrix*. Rose (1963) has suggested that circulating antibodies may be important only when combined with a local factor such as a cellular response. Various investigators have studied the role of cellular immunity as the protective factor against coccidiosis. Becker (1935) suggested that the localized response may be due to "sensitized macrophages". Van Doorninck and Becker (1957), Doran (1966c), and Challey and Burns (1959) in their studies on the transport of

sporozoites by the macrophages, have speculated on the role these cells play in the immune response mechanism. Horton-Smith (1963) and Pierce et al (1962), have demonstrated the presence of numerous pyroninophilic cells - resembling plasma cells - and also leukocytes in the caeca of immunized birds after challenge with E. tenella. In discussing these plasma-like cells, Horton-Smith suggests that the macrophages may ingest not only sporozoites but also eosinophils which have come in contact with this antigen. Macrophages may then transform into the plasma cells and produce antibody which destroys the sporozoite and inhibits further growth. However, to date, the exact mechanism of the immune response to coccidiosis has not been elucidated.

III. EXPERIMENTAL METHODS AND DESIGN

A. Experimental Diets

1. Basal Diet No. 1: 21% protein.

Ingredients	Percent in ration
Ground wheat	74.5
Soybean meal	12.0
Herring meal	8.0
Dried distillers solubles	2.0
Bonemeal	2.0
Limestone	1.0
Iodized salt	0.5
	Amount per kilogram
Manganese sulfate	12.5 grams
Riboflavin	0.33 grams
Vitamin D_3	440 I.C.U.

2. Experimental Diet No. 2:

Basal Diet No. 1 plus 440 I.U. vitamin A* per kilogram of feed.

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* Vitamin A palmitate - Rovemix A-325. Hoffman-La Roche Ltd.

3. Experimental Diet No. 3:

Basal Diet No. 1 plus 4400 I.U. vitamin A* per kilogram of feed.

B. Brooding

One hundred day-old White Leghorn cockerels were placed in electrically heated battery brooders with wire screen floors. For the first 4 days the chicks were all fed Basal Diet No. 1 containing no supplementary vitamin A. At 4 days of age the chicks were wing banded, distributed randomly into 4 sections of the brooder, and fed their experimental diets. The 2 top sections of the brooder were used for chicks on Diet No. 2, low vitamin A; and the 2 lower sections were used for chicks on the normal vitamin A diet, No. 3. This arrangement prevented the chicks on the low vitamin A diet accidently getting a higher intake of vitamin A from spilled feed. During the first 2 weeks in the brooder any weak or deformed chicks were killed. Both feed and water were available at all times.

The chicks were weighed at 18 days of age, and the average for each group was:

Diet No. 2: 171 grams

Diet No. 3: 170 grams

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* Vitamin A palmitate - Rovemix A-325. Hoffman-La Roche Ltd.

At this time the growing chicks were redistributed into 6 sections of the brooder to give 15-16 chicks to each section instead of 25. All the birds on the low vitamin A diet were aligned on one side of the battery brooder, and the birds on the normal vitamin A on the other. This arrangement eliminated any chance of feed contamination from one group to the other, and yet standardized brooder positions.

At 4 weeks of age the chicks were again weighed and had the following averages per group:

Diet No. 2: 294 grams

Diet No. 3: 296 grams

The birds were then transferred to the experimental room and placed in non-heated wire batteries.

Brooder Quarantine Methods

During the time the chicks were in the brooder room, strict quarantine methods were practised to ensure that they remained coccidia-free. These included:

1. Before the chicks were introduced, the brooder and troughs were cleaned, sterilized, and re-painted with aluminum paint to eliminate any previous contamination.
2. The floor of the room, tables, and sink were scrubbed with undiluted bleach (sodium hypochlorite) before chick introduction, and twice a week thereafter to

destroy any oocysts which might have been carried in.

3. All cleaning equipment such as brooms, scrapers, sponges, plus containers used in the brooder room were bought new and never removed from the room.
4. Every effort was made to keep the room fly-free. An insecticide bait was present to kill any flies that did enter.
5. Only one person, the author, tended the chicks during the course of the entire experiment.

To ensure that these precautions were satisfactory, fecal samples were taken periodically and examined for the presence of oocysts. It would appear from the negative findings that these procedures were successful.

C. Experimental Design

When the birds were transferred to the experimental room at 4 weeks of age, they were divided into 3 weight categories: light, average, or heavy, based on the average weight of 295 grams. Each of the 2 dietary groups was subdivided into 4, each subgroup containing 10 birds: 3 light, 4 average, and 3 heavy. These groups were designated as follows:

Low vitamin A Diet No. 2: Groups A, B, C, D.

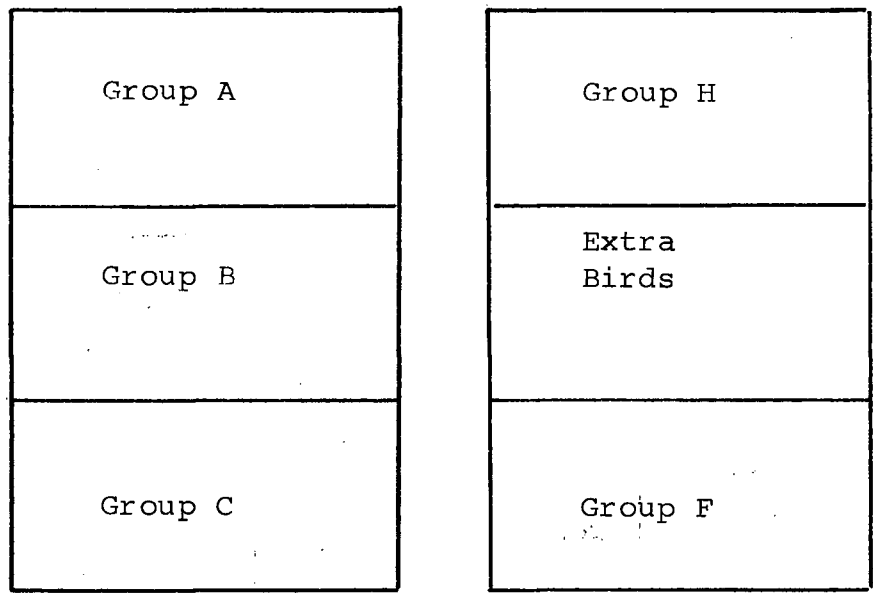
Normal vitamin A Diet No. 3: Groups E, F, G, H.

Extra birds were held in case they were needed for replacement, and were maintained on their starting diets.

The birds were caged in 3 batteries, each containing 9 sections. The birds were kept 3 and 4 to a section for most of the experimental work. As the experiments progressed, and the number of birds diminished, it was possible to allow the older birds more space, placing 2 to a section. The arrangement of the birds in the batteries is shown in Figure 1.

To ensure that the control groups were kept completely free from infection until challenge, the following precautions were undertaken:

1. The non-infected control groups C, D, and G were kept in a separate battery (No. 3) during the course of the first experiment. Control group H was placed in the top row of battery No. 2, and was separated from the infected group by a buffer layer of extra birds. Modifications of this plan were made for the second experiment.
2. The bags of feed for each diet were divided into 2 lots. One was used for the infected group and the other for the controls.
3. The control birds were always fed, watered, and cleaned first. All utensils used for the control groups were



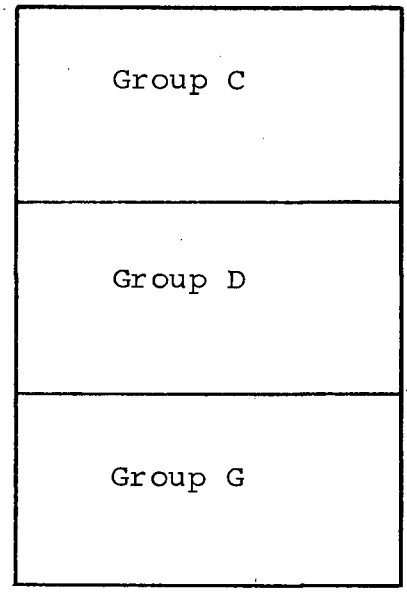


Figure 1. Arrangement of chicks in batteries for Experiment No. 1.

separated from those used for the infected groups. A separate coat was worn for tending each group.

4. No personnel, other than the author, were permitted in the experimental room during the entire course of the experiments.

Periodic checks of the feces from the non-infected groups revealed no evidence of oocysts.

D. Methods

1. Oocyst determinations and techniques

The culture of E. acervulina used in these experiments was received from the Norwich Pharmacal Co. in August, 1968 and was termed American Cyanamid strain. Originally it was intended to use this culture only to infect birds for oocyst production, but numerous trials on young birds did not produce enough oocysts for a large scale infection, and so most of the original culture was used for Experiment 1. The oocysts passed by the birds in this experiment were collected and used for Experiment 2, and the oocysts from Experiment 2 were harvested and used for Experiment 3.

a) Calibration of oocyst numbers for inoculation

(1) Total number of oocysts required for the experiment is determined.

(2) The number of oocysts present in 1 ml. of the stock culture is calculated by diluting this amount in 500 ml. water, for example; counting the number in 1 cu. mm. by use of a hemocytometer; multiplying this figure by 1000 to give the number of oocyst in 1 ml.; and multiplying this amount by 500 to give the number in 1 ml. of original culture.

(3) The amount of culture required for the experiment is then removed, centrifuged and washed several times, and resuspended in physiological saline (0.9%) to give a final dilution of about 1 million oocyst/ml. The washing is necessary to remove the potassium dichromate in which the oocysts are suspended.

b) Collection and sporulation of oocysts

As previously stated, oocysts were harvested from Experiments 1 and 2 to be used for inoculum in the succeeding experiments. The procedures for collection and sporulation were subjected to much experimentation to determine the best method for the preparation of the large numbers of oocysts required for these experiments. The usual methods recommended in literature utilized some type of flotation for the collection of oocysts from the feces. However, it was found that none of the methods

tried yielded a high enough percentage of oocysts from the fecal mass, and so the following modification was devised to recover the maximum number of oocysts.

(1) The infected feces were collected and mixed with enough 2% potassium dichromate, in a shallow pan, to give a slushy mixture.

(2) This mixture was covered with a damp cloth and left at room temperature (26°C) for 48 hours to allow sporulation of the oocysts. There was a great deal of difficulty encountered at this stage. The degree of sporulation was extremely low, averaging about 20% in the early efforts. Incubation at higher temperatures did not prove effective, nor did constant agitation, bubbling in oxygen, or other modifications. Storage of the feces in the refrigerator was found to be the principal factor limiting sporulation, and it was found that by processing feces immediately after collection a much higher rate of sporulation could be acquired. However, the highest sporulation effected never extended beyond 50-60%.

(3) There was no evidence that additional sporulation occurred after 48 hours, so at this time the mixture was passed in sequence through 2 sieves, 30 and 100 meshes per linear inch respectively, using a jet of tap water to force it through.

(4) The collected washings were put into 1000 ml. graduates, and water added, if required, to completely fill the container. The suspension was allowed to stand overnight, and then the supernatent was decanted and the sediment mixed with 1000 ml. tap water and again left to settle out. This operation was repeated 2 or 3 times to remove as much extraneous debris as possible from the oocysts.

(5) Following the last decanting, the sediment was mixed with 2% potassium dichromate and stored in the refrigerator until required.

This method gave the maximum number of oocysts but did result in a culture heavily contaminated with fecal material. However, no ill effects were observed which could be attributed to this contamination, and as the culture was used shortly after collection, the oocysts did not degenerate from the bacterial contamination.

c) Calculation of the daily oocyst production per bird

The number of oocysts passed daily by an infected bird is one criterion of the bird's response to the disease. In these experiments, the total fecal output from each inoculated group was collected daily, starting on the 4th day after inoculation and continuing for 7 days. After this time the collections were made every second day for the next 4 to 8 days depending on conditions. The total number of oocysts per bird per day was calculated by the following methods:

(1) The total daily feces from each group was put into a container and weighed. If the samples were not processed immediately, they were mixed with enough 2% potassium dichromate to keep them moist and then placed in the refrigerator until processed.

(2) The total sample was then thoroughly mixed in a basin and a 10 gram sample removed. If the feces had been mixed previously with potassium dichromate a sample calculated to be equivalent to 10 grams of untreated feces was used.

(3) This 10 gram aliquot was then mixed with 500 cc of tap water in a Waring blender and agitated at full speed for 1½ minutes.

(4) Immediately following homogenization, 6 separate 1 ml. samples of the suspension were removed and placed in a test tube. This sample was mixed 10 times with a Pasteur pipette and one drop was then removed and placed in one chamber of the hemocytometer. This procedure was repeated to fill the second chamber.

(5) The total number of oocysts present in the 5 large squares of each chamber were counted. If the 2 chambers showed more than a 10% discrepancy in the counts, a further 2 samples were counted and an average taken.

(6) The total number of oocysts per sample was calculated as follows:

Average hemocytometer count	= Y (number in 1 cu. mm)
Number in 1 ml.	= 1000Y
Number in 500 ml.	= 500 X 1000Y

The number of oocysts in 500 ml. is equivalent to the number in 10 grams of feces, and from this figure the total number of oocysts per group could be determined. This amount was divided by the number of birds in the group to give the oocysts/bird/day.

Using this method of calibration, each oocyst counted in the hemocytometer was equivalent to 500,000 oocysts, or 50,000 per gram of feces. Therefore, if the numbers were below this figure per gram, the sample would appear to be negative. Due to the large amount of extraneous matter present in the fecal samples, a smaller dilution factor was not possible when using the hemocytometer. Therefore, to avoid the false assumption that a sample having below 50,000 oocysts per gram was negative, any sample showing one or no oocysts was subjected to the McMaster Counting Technique as follows:

(1) The total daily fecal mass per group was well mixed and a 10 gram aliquot removed and mixed in a beaker with 95 ml. of a saturated solution of sodium chloride.

(2) Using a Pasteur pipette, the 0.15 ml. chamber of a McMaster slide was filled with the coarse suspension. The oocysts floated free of the debris and pressed against the bottom of the scored cover slip where they could be counted.

(3) The average of 2 counts was taken to give the total number of oocysts in 0.15 ml. The total number of oocysts in 10 grams was calculated as follows:

Total oocysts in 0.15 ml. = Y

Total in 95cc (10 grams) = Y X 100 X 7

Using this technique, each oocyst counted was equivalent to 700 oocysts, or 70 per gram of feces.

d) Calculation of the reproductive index

The reproductive index or reproductive potential is a measure of immunity or resistance offered by the bird against a coccidial infection. As such it is a useful tool in the assessment of the host's reaction and was calculated for each bird at the conclusion of each experiment. It is calculated as follows:

$$R.I. = \frac{\text{Total number of oocysts passed}}{\text{Total number of oocysts inoculated}}$$

2. Histological techniques

a) Fixation

Immediately after a bird was killed, the duodenal loop was removed and placed in Bouin's fixative. The formula for this solution is:

Saturated picric acid	75cc
Formalin	25cc
Acetic acid	5cc

b) Dehydration and embedding

(1) A 3-5 mm section of the duodenum, distal to the turn of the loop, was removed and placed in a mesh plastic capsule (Tissue Tek) for processing.

(2) Dehydration started with 3 changes of 70% ethyl alcohol, within a 48 hour period, to remove as much picric acid as possible from the tissue. This was followed by 2 hours or more in 95% alcohol; 2 changes in 99% alcohol of one hour each; and clearing in 2 changes of xylol, one hour each.

(3) The dehydrated tissue was then transferred to 3 changes of wax in a 60° oven, and embedded in "Tissue Tek" embedding rings.

c) Sectioning

The sections were cut at 5 microns using a Leitz micrometer. The ribbons were floated on water, and the sections affixed, 3-4 per slide, using egg albumen or gelatin.

d) Staining

A great deal of experimentation was undertaken to find a differential stain which would clearly show the sporozoites in the tissue. Pattillo (1959) recommended PAS Feulgen for this purpose; Clarkson (1958) and Shortt and Cooper (1948), a Giemsa modification; Horton-Smith and co-workers (1963) recommended McFarlane's (1944) Picro-Mallory stain; and Challey and Burns (1959) used Groat's hematoxylin followed by Shorr's stain. All these recommendations were tried in addition to Groat's

Tetrachrome Stain. None of these methods proved very satisfactory and eventually 2 methods were used exclusively. These were a standard hematoxylin and eosin technique and a modified Groat's Tetrachrome Stain. The standard method followed was:

(1) The sections were dewaxed through 2 changes of xylol and hydrated through 99%, 95% and 70% alcohols.

(2) Stained in Harris' Hematoxylin for 5-10 minutes, then decolourized in acid alcohol and blued in ammonia water.

(3) Counterstained in either aqueous eosin (standard H and E), or in Biebrich Scarlet-Orange II stain (Groat's Tetrachrome).

(4) Washed, dehydrated, and cleared in xylol.

(5) The sections were mounted with "Pro-Tex" mounting medium and covered with 22mm by 50mm coverslip.

With both these stains the refractile vacuole in the sporozoite was stained red, making it easy to identify. However, although the Groat's modification gave a better differentiation of blood cells, it tended to overstain, and therefore the standard H and E was the recommended method.

E. Experiments

The 8 groups of birds were used in 3 different experiments. The first experiment utilized 5-week-old birds, the

second 9-week-old birds, and the third 19-week-old birds.

1. Experiment Number 1

a) Object

a) To assess the response of 5-week-old chicks, on 2 levels of vitamin A, to a primary infection with E. acervulina.

b) To immunize half the chicks of each dietary group against E. acervulina.

b) Design

The groups of chicks were arranged according to the previously discussed experimental design. Four groups, each consisting of 10 birds, were infected. The groups were as follows:

Low vitamin A diet: Groups A and B infected
Groups C and D controls

Normal Vitamin A diet: Groups E and F infected
Groups G and H controls.

c) Inoculation

The culture used for this first experiment was the original culture of sporulated E. acervulina oocysts received from Norwich Laboratories. An inoculum was calibrated to contain 3 million oocysts per ml. of physiological saline, and each bird from groups A, B, E, and F was inoculated orally with

1.0 ml. - 3 million oocysts. This approximated 8000 oocysts per gram of body weight. The birds were starved 24 hours before inoculation.

d) Methods

(1) Strict hygienic and quarantine techniques were employed to ensure that the control groups remained free of infection. These were discussed under Experimental Methods and Design - Section C.

(2) The cages of the infected birds were cleaned every second day, from day 4 to the end of the experiment, to ensure there was no re-infection of the birds.

(3) Fecal samples were collected daily and the number of oocysts per bird per day was calculated.

(4) The infected birds were weighed at the time of inoculation, on day 4, and on every second day following this for about 20 days.

(5) The non-infected birds were weighed weekly.

(6) In this experiment, and in the 2 that followed, the weights between groups were subjected to an analysis of variance to determine

if the differences were statistically significant.

2. Experiment Number 2

a) Object

To compare, in 9-week-old birds raised on 2 levels of vitamin A:

(1) The degree of acquired immunity retained 4 weeks after a primary infection with E. acervulina.

(2) The early tissue stages of E. acervulina in immune and non-immune chicks.

(3) The response of susceptible 9-week-old chicks to a primary infection with E. acervulina.

b) Design

Before this experiment was conducted, the groups of birds were re-arranged in numbers, keeping the weights as uniform as possible. The groups were adapted for this experiment as follows:

Low vitamin A diet:

Group A - 10 birds - To be given a second infection.

Group B - 8 birds - Non-infected immune controls.

Group C - 10 birds - To be given a primary infection.

Group D - 9 birds - Non-infected non-immune controls.

Normal vitamin A diet:

Group E - 11 birds - To be given a second infection.

Group F - 9 birds - Non-infected immune controls.

Group G - 11 birds - To be given a primary infection.

Group H - 9 birds - Non-infected non-immune controls.

The infection of groups A and E would determine the degree of immunization retained 4 weeks after a primary infection; and the infection of groups C and G would examine the response of these 9-week-old birds to a primary infection.

c) Inoculation

The culture consisted of sporulated oocysts of E. acervulina harvested 4 weeks earlier from Experiment No. 1. The inoculum was calibrated to give 5 million oocysts per ml. of saline and the birds were infected per os with 2 ml., giving 10 million oocysts per bird. This averaged out to 11,000 oocysts per gram of body weight.

d) Methods

(1) Three birds from each group were killed 3 hours after inoculation, and another 3 birds were killed at 48 hours. The duodenum was removed from each bird and fixed in Bouin's solution.

(2) Pooled fecal samples from each group were taken daily and processed to determine the total number of oocysts passed per bird per day.

(3) Standard hygienic practises were observed to prevent infection of the control groups.

(4) The groups B, D, F, and H, were held for 10 weeks following Experiment No. 2, and during this period they were weighed once a week.

(5) The remaining birds in Groups A, E, C, and G were eliminated 3 weeks after inoculation when Experiment No. 2 was terminated.

3. Experiment Number 3

a) Object

To compare the response of 19-week-old birds, raised on 2 levels of dietary vitamin A, to an infection with E. acervulina in order to determine:

(1) The degree of immunity retained by birds 14 weeks after a primary infection.

(2) The response of older birds to a heavy primary infection.

b) Design

All the remaining groups were infected in this 3rd experiment. They were:

Low vitamin A diet:

Group B - 7 birds - primary infection 14 weeks earlier.

Group D - 8 birds - No previous infection.

Normal vitamin A diet:

Group F - 8 birds - primary infection 14 weeks earlier.

Group H - 8 birds - No previous infection.

c) Inoculation

The oocysts used for this experiment were harvested from Experiment No. 2. They were stored in the usual manner and re-suspended in physiological saline to give 2.5 million oocysts ml. All the birds were inoculated orally with 25 million oocysts, a dose of 10 ml. per bird. This was roughly equivalent to 14,000 oocysts per gram of body weight.

d) Methods

(1) As there were no non-infected controls in this experiment, the hygienic techniques were not as strict as in the previous experiments. However, precautions were maintained to omit extraneous contamination and to prevent re-infection from the contaminated feces.

(2) Two birds from each group were killed on the 4th day after inoculation. These birds were autopsied and clinical evidence of the disease was noted. The duodenal loop, and any other

areas of the intestine showing gross lesions, were removed and fixed in Bouin's solution for sectioning and histological examination.

(3) Daily pooled fecal samples from each group were taken and the number of oocysts determined per bird.

(4) A record of weights was maintained following the routine of the previous two experiments.

IV. RESULTS

A. Experiment No. 1

This experiment examined the response of 5-week-old chicks, on low and normal levels of vitamin A, to a primary infection with E. acervulina.

Clinically, the birds on low vitamin A rations (AB) were more affected by the disease than the birds on normal vitamin A rations (EF). Both groups of birds began to show symptoms of the disease at day 3, when a slight drop in feed consumption was observed. A watery diarrhea was apparent by day 4, with scanty mucoid droppings characteristically stained green. Anorexia and adipsia were pronounced in both groups on day 4 and 5, and the anorexia continued in the low vitamin A group until about day 14. The normal vitamin A group (EF) regained their appetites by day 7-8. The droppings of the low vitamin A group (AB) were scantier and more watery than group EF throughout the acute stage of the disease (days 4-6), and did not return to normal consistency until day 12, compared to day 7 for group EF. There was no mortality in the normal vitamin A group, but 2 birds died in group AB. The first died on day 10 and showed a 27 percent loss in body weight at that time, and the second died on day 12 after losing 22 percent body weight. Neither bird revealed

severe intestinal pathology, but both were very dehydrated and atonic.

The low vitamin A group passed a total average of 615 million oocysts per bird between days 4 and 14, giving a reproductive index of 205. The birds on normal dietary levels of vitamin A had a total average production of 360 million oocysts and a R.I. of 120 for the same period. As shown in Table 1 both groups had a peak oocyst production on day 5, but group AB maintained a higher daily level throughout the course of the experiment. Periodic fecal examinations for the presence of oocysts were made during the 3rd week, and it was found that group EF was essentially negative by day 18, but the birds of group AB were still passing several million oocysts/bird/day. However, all the birds were negative by the time the 2nd experiment was instigated at day 28.

Table II shows the total weights of the 4 experimental groups during the course of Experiment No. 1. The groups all had similar weights at the start of the experiment but by day 4, the two infected groups of birds were significantly lower in weight ($P < .01$) than the controls. The infected low vitamin A group of birds (AB) were an average of 28 grams lighter in weight than group EF on day 6 and this difference increased to 80 grams by day 14. There was no significant difference in

weight between the 2 control groups throughout the experiment. The low vitamin A control group was about 4 percent less in average total weight than the other 3 groups at the start of the experiment, and maintained this difference with the other control group until the end. However there was a marked difference between the 2 infected groups. There was a significant difference in mean weight between day 8 and 18 ($P < .01$) which although diminishing, was maintained until day 21 ($P < .05$). By day 14 of the experiment the low vitamin A group had lost 25 percent of its body weight compared to its control, while the normal vitamin A group had lost only 16 percent as illustrated in Figure 2.

It took several weeks before the weights of the infected groups were statistically equal to those of the control groups as shown in Table IV. Figure 3 graphically compares the weights of infected groups B and F, and their non-infected controls, D and H respectively, from November 1st to February 23rd. During this 4 month period the non-infected controls did not show a significant difference in weight. Groups B and F were infected at 5 weeks, and at 6 weeks showed a significant ($P < .01$) difference in weight from their control groups. This difference was maintained in the normal vitamin A groups, F and H, until 4 weeks

after the date of inoculation. The low vitamin A groups B and D showed a significant difference in the total weights of the birds until 6 weeks after inoculation. However, there was a significant difference in weight ($P < .05$) between groups B and H until 9 weeks after inoculation at which time the birds were 14 weeks old.

The weight changes which occurred during the 2 weeks following infection are shown in Table III. This table gives the weight gained or lost in 2 day intervals from day 4 to 14 of infection, plus the infected groups weight gains compared to the controls. During the acute stage of infection, between days 4 and 6, the low vitamin A group lost an average of 25 grams per bird, while the normal vitamin A group lost only 4 grams. Between days 6 and 8 group AB still showed a slight loss in weight while group EF had started to gain and by day 14 both groups were showing similar weight increases. However, the low vitamin A group showed an average gain of only 64 grams per bird during the 14 days, compared to 145 grams for the normal vitamin A group. This was significantly different at the .01 probability level. The controls showed no significant difference in the total average weight gained over this same period. Figure 4 illustrates this difference in weight gains, and gives a graphic picture of the deleterious effects of a low vitamin A

diet. The chicks on the low vitamin A ration had a weight gain 63 percent lower than that of their control group, while the infected birds on normal vitamin A rations were only 30 percent lower in weight gains from their control.

A comparison of the different reactions to an infection with E. acervulina in 5-week-old birds on normal and low vitamin A rations, shows that the severity of the disease is almost doubled in the birds on low dietary vitamin A. The performance index has been used by Stock; Stevenson; and Hymas (1967), and Stevenson (1969), to evaluate the total effects of coccidiosis in different groups of birds. It encompasses the weight gain, mortality and oocyst production, and although an artificial method of comparison, it does give an indication of total effect. It was modified in this investigation to give some standard of comparison between experiments, and the formula devised was: average gain + percentage survival - $\frac{\text{oocyst production in millions}}{10}$. Using this formula the P.I. of the low vitamin A group (AB) was 64 + 90 - 61 = 93, and that of the normal vitamin A group (EF) was 145 + 100 - 36 = 209. Thus it can be seen that by taking mortality into account plus the severity of the disease in the survivors, the low vitamin A group was over twice as severely affected by the infection with E. acervulina as was the normal vitamin A group.

TABLE I

AVERAGE DAILY OOCYST PRODUCTION IN MILLIONS IN 5-WEEK-OLD CHICKS, ON 2 DIETARY LEVELS OF VITAMIN A, FOLLOWING INFECTION WITH 3 MILLION OOCYSTS OF *E. ACERVULINA* PER BIRD

Day	Low vitamin A diet (AB)	Normal vitamin A diet (EF)
4	55	20
5	125	125
6	95	60
7	85	50
8	75	30
9	70	30
10	55 (19)*	25
12	35 (18)	15
14	20 (18)	5
Total oocysts per bird	615	360
Reproductive Index	205	120

* Surviving birds out of 20.

TABLE II

MEAN WEIGHT IN GRAMS OF 5-WEEK-OLD CHICKS, ON 2 DIETARY LEVELS OF VITAMIN A, AFTER INFECTION WITH 3 MILLION OOCYSTS OF E. ACERVULINA COMPARED TO NON-INFECTED CONTROLS

Day	Infected birds			Non-infected birds	
	Low vitamin A diet (AB)		Normal vitamin A diet (EF)	Low A diet	Normal A diet
0	396		397	380	398
2	434		436		
4	409		416	465	486
6	383		412		
8	378	**	433		
10	397 (19)a	**	455		
12	425 (18)	**	499		
14	460 (18)	**	542	601	632
16	497 (18)	**	570		
18	538 (18)	**	621		
20	581 (18)	*	662	737	786

a. birds surviving out of 20.

* significant difference in weight ($P < .05$) between AB and EF.

** highly significant difference in weight ($P < .01$) between groups AB and EF.

TABLE III

DAILY MEAN WEIGHT CHANGES IN GRAMS OF 5-WEEK-OLD CHICKS, ON 2 DIETARY LEVELS OF VITAMIN A, FOLLOWING INFECTION WITH 3 MILLION OOCYSTS OF *E. ACERVULINA*

Between Days	Low vitamin A ration		Normal vitamin A ration	
	Infected (AB)	Control (CD)	Infected (EF)	Control (GH)
0-4	+15	+85	+19	+88
4-6	-25		- 4	
6-8	- 3		+21	
8-10	+19		+22	
10-12	+27		+45	
12-14	+36		+43	
Total weight gain in 2 weeks*	+64c	+221a	+145b	+234a
Grams per day of gain	4.5	+16	10.5	+17

* means showing the same letter did not differ significantly ($P < .01$).

TABLE IV

TOTAL MEAN WEIGHT IN GRAMS, OVER A 4 MONTH PERIOD, OF 4 GROUPS OF CHICKENS ON 2 DIETARY LEVELS OF VITAMIN A, AND WITH ONE GROUP ON EACH LEVEL INFECTED AT 5 WEEKS OF AGE WITH 3 MILLION OOCYSTS OF E. ACERVULINA

Age - weeks	Low vitamin A		Normal vitamin A		
	Infected B	Control D	Infected F	control H	
2	180	178	175	171	*
5 - infected	377	369	387	392	*
6	377	470	420	500	
8	564	741	674	802	
10	820	997	984	1076	
12	1066	1221	1188	1293	
14	1348	1431	1472	1567	*
16	1520	1579	1660	1690	*
18	1695	1695	1818	1848	*

* no significant difference ($P < .05$) between groups B, D, F, H.

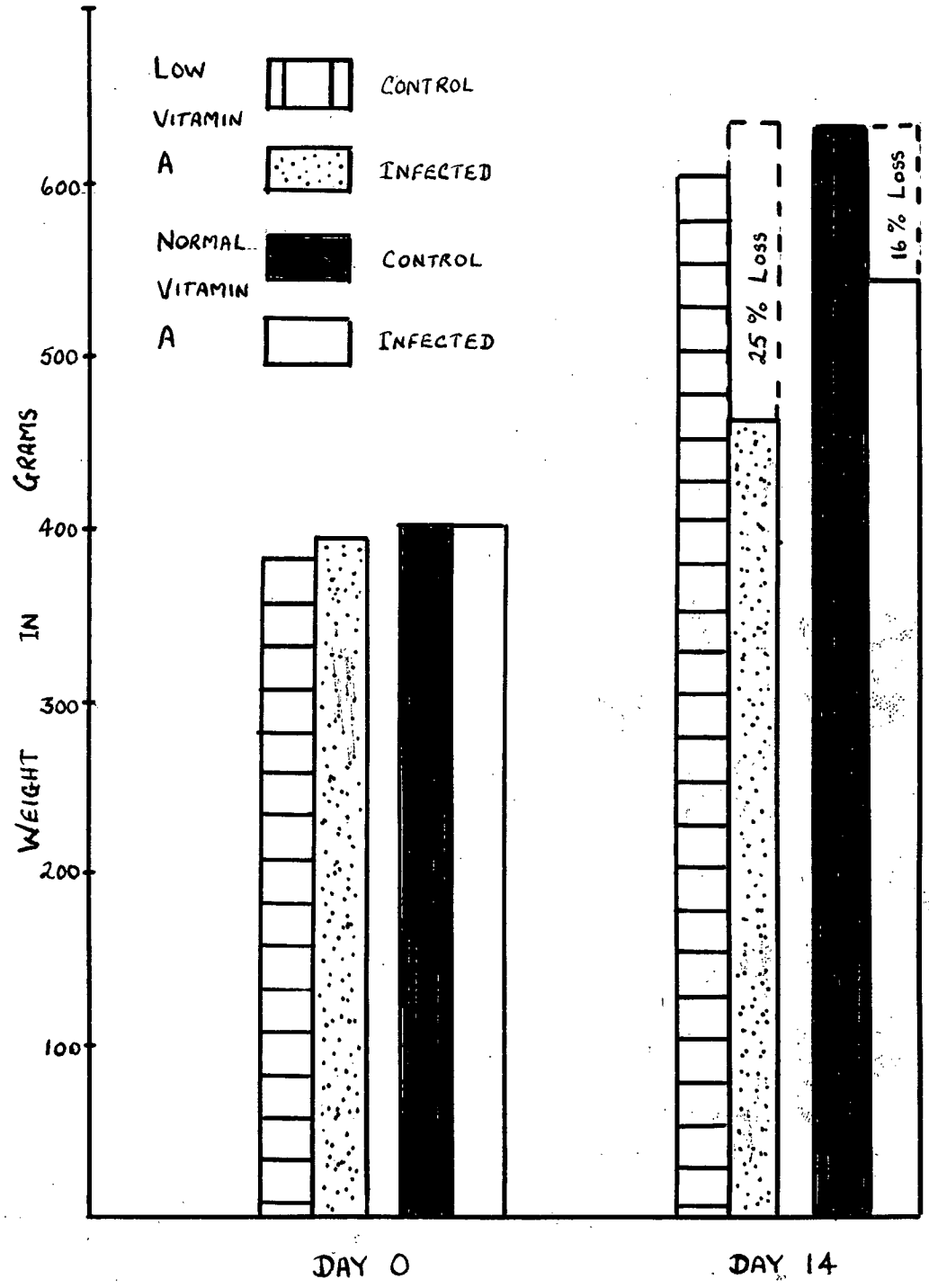
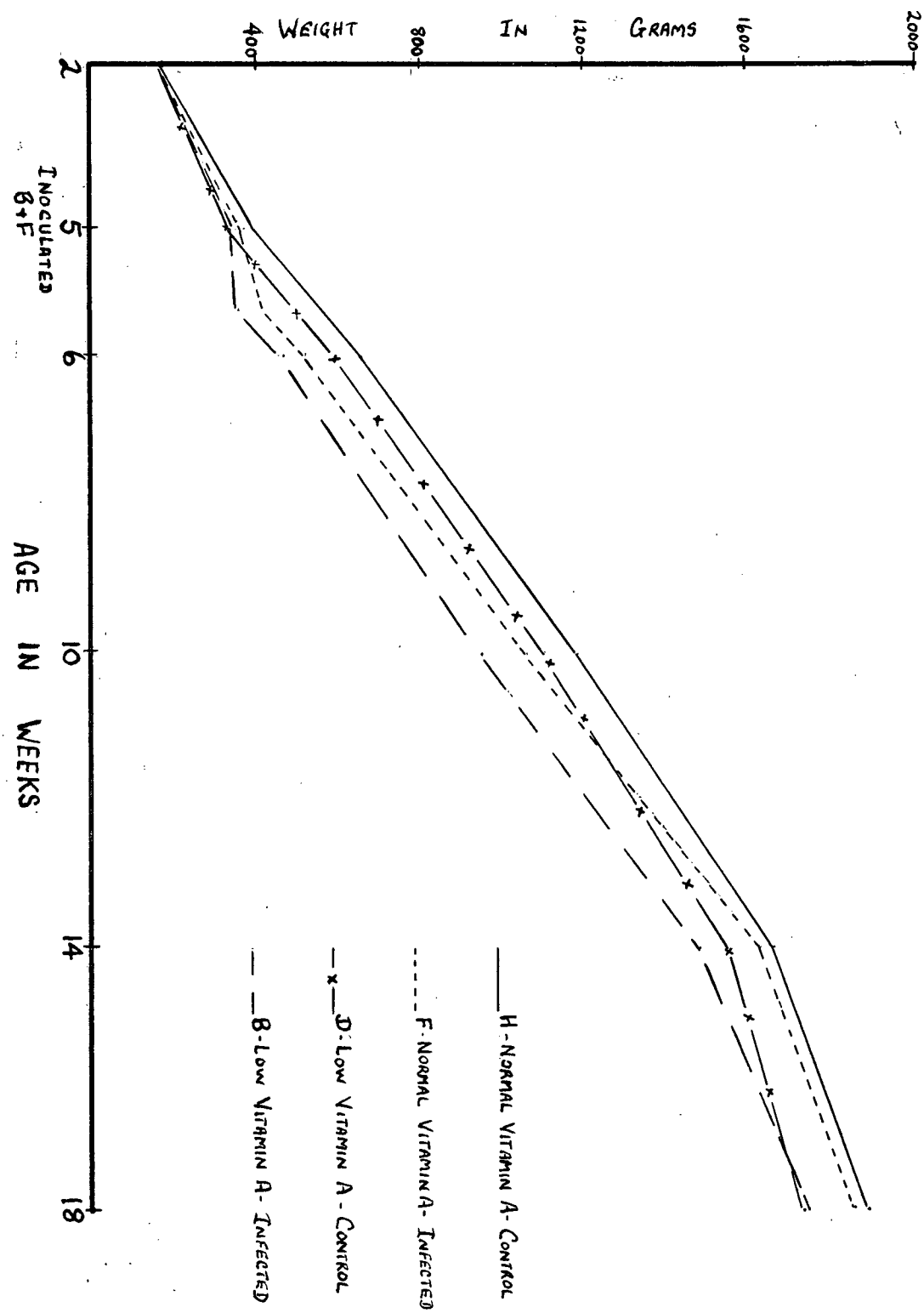


Figure 2. The effect of dietary vitamin A level on the mean weight of 5-week-old chicks following infection with E. acervulina

Figure 3. A comparison of the mean weights of infected and non-infected chicks, on 2 dietary levels of vitamin A, over a 4 month period



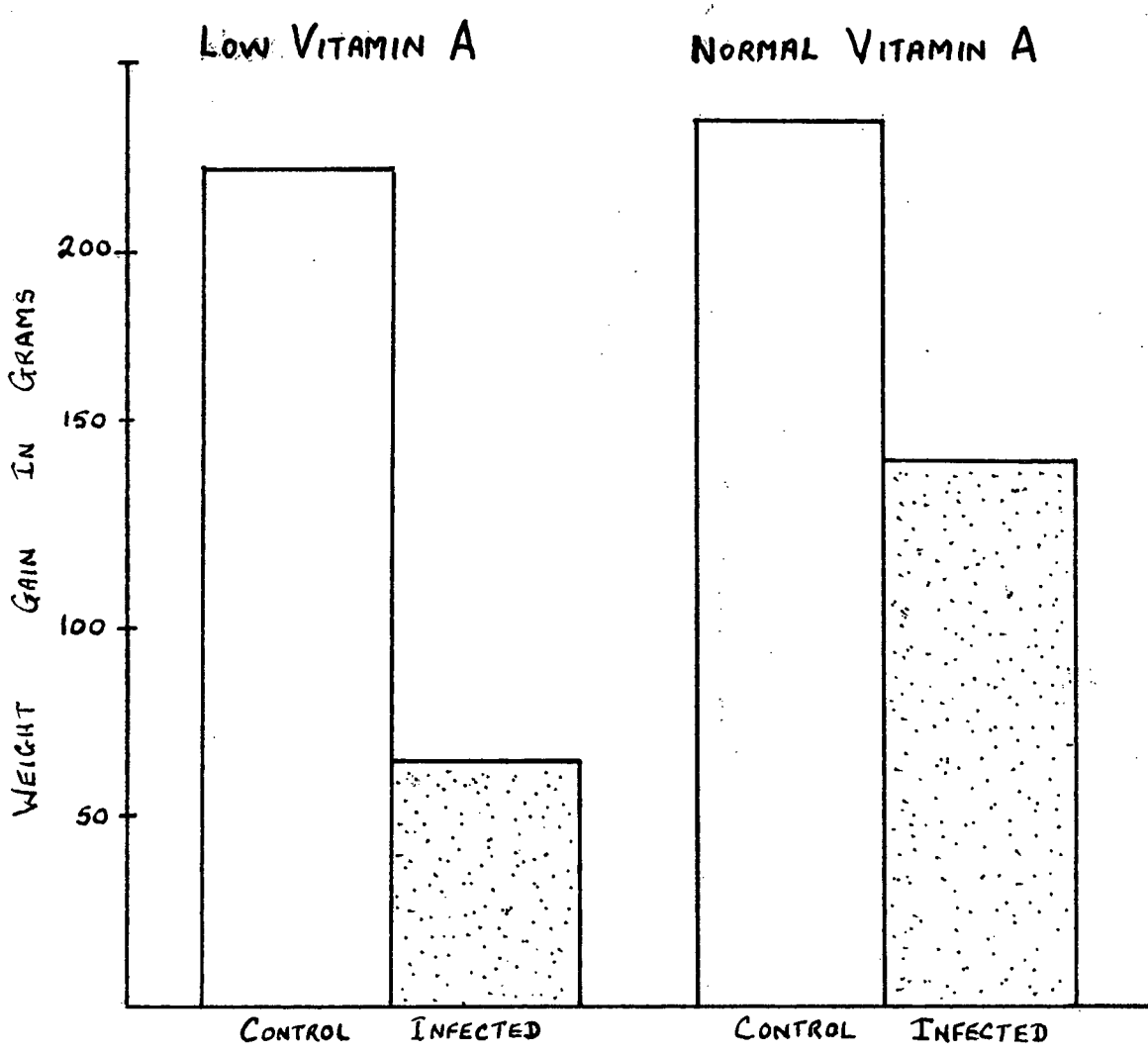


Figure 4. The effect of dietary vitamin A level on the 2 week weight gain of 5-week-old chicks following infection with E. acervulina

B. Experiment No. 2

The primary purpose of this experiment was to study the early endogenous forms of E. acervulina in immune and non-immune 9-week-old chicks receiving 2 different levels of vitamin A in the ration. Six birds from each group were killed for histological examination of the intestines, leaving only 4-5 birds per group, and this small experimental number may have resulted in some of the clinical results appearing less significant than they actually were.

1. Clinical Results

Clinically, the immune groups A and E, showed little response to their second infection. A slight anorexia was noted on days 4-6 in both groups, and although the droppings of group E were slightly watery on day 5, there was no other evidence of abnormal feces. The non-immune groups, C and G, receiving their primary infection with E. acervulina, manifested the typical symptoms of the disease on days 4-8, but these symptoms were not as severe as in the younger birds of Experiment 1. There was no mortality in either of these groups, but both groups had a marked anorexia and watery, scant droppings on days 4-6. Diarrhea was most severe on day 5, especially in the low vitamin A group (C). The birds on normal levels of vitamin A (G) regained their appetites more quickly

than those in group C, and showed less evidence of diarrhea after day 5.

A comparison of the oocyst numbers passed in the course of the disease is shown in Table V. The protective effects of immunity from a primary infection can be seen in groups A and E. There was no evidence of the disease on day 4, and day 5 showed a low peak for both groups. The low vitamin A group (A) appeared to have a more solid immunity as the birds passed an average of only 6 million oocysts per bird in the 14 days following inoculation, compared to 70 million for group E. The more rapid drop in daily oocyst production in group A, compared to group E, is also indicative of a better immune response. Groups C and G, receiving their primary infection at 9 weeks, produced high numbers of oocysts, but unlike the 4-week-old birds of Experiment 1, the peak production was on day 4 as well as 5. The daily course of oocyst production duplicated the findings of Experiment 1. The low vitamin A group maintained a high daily level, while the normal vitamin A group peaked on day 4 and dropped abruptly to lower and more rapidly decreasing levels. The relative totals of oocysts between the two groups on a primary infection also followed the results of Experiment 1. The low dietary vitamin A group birds had twice the number of oocysts, and twice the reproductive

index, of the birds from the normal vitamin A group.

Table VI compares the total average weight, over a period of 2 weeks, of the birds immunized in Experiment 1 when half the group is re-infected with a large number of oocysts. It can be seen that the second infection did not affect the average weight in either the low or normal dietary vitamin A groups. In fact, as shown by the total weight gains over this same period in Table IX, the infection even appeared to accelerate weight gains in the low vitamin A group (A).

The 2 groups of birds, C and G, subjected to a primary infection of E. acervulina at 9 weeks, did not suffer the weight losses of their 4-week-old counterparts in Experiment 1. As shown in Table VII, the low vitamin A group (C) lost an average of 19 percent body weight, in the 14 days following infection, compared to the non-infected controls. The normal vitamin A group (G) showed only a 4 percent loss in body weight. Table VIII shows the weight gains and losses during these 14 days. It can be seen that the maximum weight loss was on day 6 in both groups, although the gain between day 0 and 4 was far below that of the two control groups. The low vitamin A group (C) showed a greater weight loss during the acute stages of the disease and did not match the recovery rate of group G. Figure 5 illustrates the difference in weight gain between these 2 groups, and demonstrates that group C had a weight gain equal to

only 26 percent of that made by its control group D, and only 30 percent of the gain made by group G. The normal vitamin A group (G) had a weight gain equal to 80 percent of its control group H.

The degree of protection afforded by immunization with a single infection of E. acervulina is shown in Table IX. Even with the small numbers of birds used in this experiment it can be seen that with low dietary levels of vitamin A, the protective properties of the immunization are well defined. The immune group A had a weight gain following infection which paralleled that of the uninfected control group D. But the non-protected group C had a weight gain 74 percent below that of the control group. With normal levels of vitamin A in the ration, the degree of protection obtained by immunization is not so dramatic. Group E had a weight gain equal to only 88 percent of the uninfected control group H, but equalled that of its immune control F. However group G, with a primary infection, had a weight gain equal to 80 percent of the uninfected control group H,so the relative degree of protection offered by immunization is not as large as seen in the low vitamin A groups.

The performance indices for the 4 infected groups in Experiment 2 are shown in Table X, and these confirm the advantage of immunization in the low vitamin A groups. The

difference in the P.I. for the 2 groups receiving a primary infection with E. acervulina (C and G), is particularly striking. The normal vitamin A (G) group has a P.I. more than 3 times that of the low vitamin A group (C).

2. Histological Examination

3 hours post inoculation:

All the sections revealed eosinophilic refractive bodies in the epithelium of the crypts and villi epithelium which were construed to be sporozoites. This presence of sporozoites in the crypts 3 hours after inoculation does not conform with most reports which state that sporozoites take 12-18 hours to migrate to the crypts. It would suggest that there is a direct invasion of the crypts with a heavy inoculation of oocysts. A very obvious finding was an accumulation of heterophils in the lamina propria. There was a very heavy concentration of these cells in groups A, E, and G, but in group C only one of the 3 birds showed sections similar to the other groups. The sections from the other 2 birds of group C revealed only a few heterophils. There did not appear to be any difference in epithelial integrity between the groups, and there was no evidence of squamous epithelium and keratinization in the groups on low vitamin A levels.

48 hours post inoculation:

In the 2 immune groups, A and E, examination of the sections showed very few trophozoites and almost no schizonts. One bird in the low vitamin A group (A) appeared to have an area of infection retained from the immunizing dose given it 4 weeks earlier. There was evidence of gametocytes and oocysts present in the tissue of one crypt and accompanying villus showing that this bird was harbouring a slight latent infection. There were sporozoites present in the crypts and villi of almost all the immune birds but very few had developed into trophozoites.

The 2 groups receiving their primary infection, C and G, showed large numbers of trophozoites and schizonts. The schizonts measured about 5 microns in diameter and were probably first generation. These were found localized in specific areas encompassing a crypt and neighbouring villi. Most of the forms were near the base of the villi, but in many areas the whole of the epithelia of the villi was parasitized with endogenous forms in varying degrees of development. There were also many sporozoites still unchanged in the tissue, and in fact one bird from the normal vitamin A diet had only sporozoites present and showed no evidence of trophozoites or schizonts. There was no definite evidence that the low dietary vitamin A groups had a greater incidence of invasion or schizont development. However,

there was an indication that a more extensive area of the intestine was invaded in the suboptimal groups, and a larger sample number may reveal something more significant.

TABLE V

AVERAGE DAILY OOCYST PRODUCTION IN MILLIONS IN 9-WEEK-OLD CHICKS ON 2 DIETARY LEVELS OF VITAMIN A, AND 2 LEVELS OF IMMUNITY, AFTER INFECTION WITH 10 MILLION OOCYSTS OF *E. ACERVULINA* PER BIRD

Day	Low vitamin A ration		Normal vitamin A ration	
	Immune (A)	Non-immune (C)	Immune (E)	Non-immune (G)
4	0	200	0	230
5	4.5	210	18	110
6	1	60	17	20
7	.02	50	25	15
8	.01	130	8	15
9	.005	110	.6	20
10	-	65	.1	5
12	-	25	.006	10
14	-	10	.005	.8
Total oocysts per bird	6	860	70	426
Reproductive Index	0.6	86	7	43

TABLE VI

AVERAGE WEIGHT IN GRAMS OF IMMUNIZED 9-WEEK-OLD CHICKS, ON 2 DIETARY LEVELS OF VITAMIN A, FOLLOWING A SECOND INFECTION WITH E. ACERVULINA

Day	Low vitamin A		Normal vitamin A	
	Infected (A)	Non-infected (B)	Infected (E)	Non-infected (F)
0	782	735*	828	860*
4	883	805*	918	945*
6	938	835	951	985
8	968	880*	989	1030*
10	1013	920*	1024	1065*
12	1032	961	1046	1108
14	1076	990*	1096	1130*

* extrapolated figures

TABLE VII

AVERAGE WEIGHT IN GRAMS OF 9-WEEK-OLD CHICKS, ON 2 DIETARY LEVELS OF VITAMIN A, FOLLOWING A PRIMARY INFECTION WITH E. ACERVULINA

Day	Low vitamin A		Normal vitamin A	
	Infected (C)	Non-infected (D)	Infected (G)	Non-infected (H)
0	924	885*	924	940*
4	952	955*	986	1025*
6	885	993	964	1056
8	874	1035*	1006	1105*
10	909	1090*	1063	1165*
12	928	1141	1105	1223
14	998	1170*	1170	1245*
Average Percentage loss in body weight compared to controls	19%		4%	

* extrapolated weights

TABLE VIII

DAILY MEAN WEIGHT CHANGES IN GRAMS OF 9-WEEK-OLD CHICKS, ON 2 DIETARY LEVELS OF VITAMIN A, FOLLOWING A PRIMARY INFECTION WITH 10 MILLION OOCYSTS OF E. ACERVULINA

Between Days	Low vitamin A levels		Normal vitamin A levels	
	Infected (C)	non-infected* (D)	Infected (G)	non-infected* (H)
0-4	+28	+70	+62	+85
4-6	-67	+38	-22	+31
6-8	-11	+42	+42	+49
8-10	+35	+55	+57	+60
10-12	+21	+51	+42	+58
12-14	+70	+29	+65	+29

* extrapolated weights

TABLE IX

TWO WEEK TOTAL WEIGHT GAIN IN GRAMS IN IMMUNE AND NON-IMMUNE 9-WEEK-OLD CHICKS, ON 2 DIETARY LEVELS OF VITAMIN A, FOLLOWING INFECTION WITH 10 MILLION OOCYSTS OF *E. ACERVULINA*

	Grams
Low vitamin A levels:	
Group A: Immune, infected:	294
B: Immune, non-infected	255
C: Non-immune, infected	74
D: Non-immune, non-infected	285
Normal vitamin A levels:	
Group E: Immune, infected	268
F: Immune, non-infected	270
G: Non-immune, infected	246
H: Non-immune, non-infected	305

TABLE X

PERFORMANCE INDEX OF 9-WEEK-OLD CHICKS ON, 2 DIETARY LEVELS OF VITAMIN A AND 2 LEVELS OF IMMUNITY, FOLLOWING INFECTION WITH 10 MILLION OOCYSTS OF *E. ACERVULINA* PER BIRD

Group	Performance Index*
A - Low vitamin A ration - secondary infection	393
C - Low vitamin A ration - primary infection	88
E - Normal vitamin A ration - secondary infection	361
G - Normal vitamin A ration - primary infection	303

* $2 \left| \text{week average weight gain} + \text{percentage survival} - \frac{\text{oocyst production}}{10} \right.$

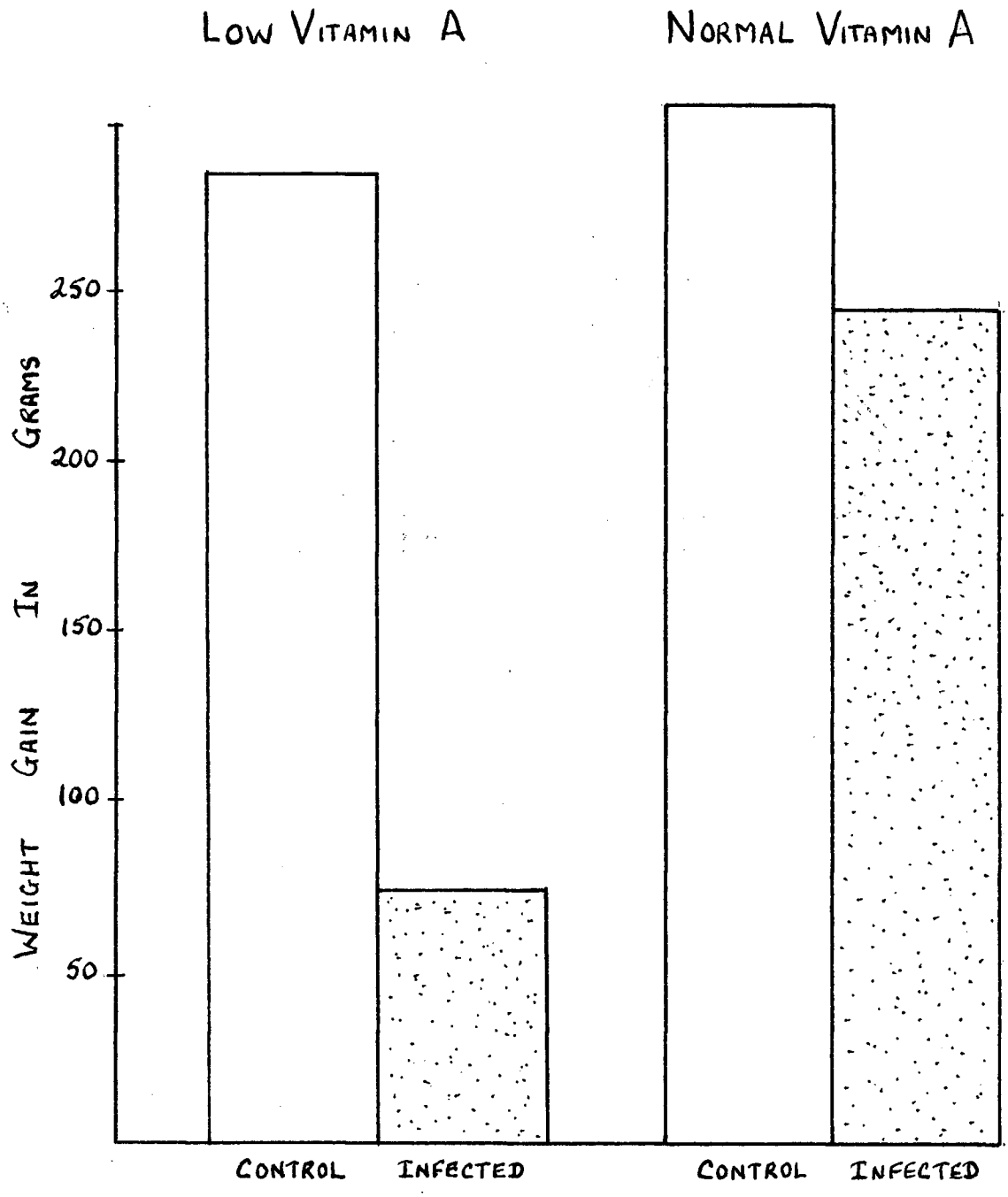


Figure 5. The effect of dietary vitamin A level on the 2 week weight gain of 9-week-old chicks following infection with E. acervulina

C. Experiment No. 3

In this experiment the birds were subjected to a massive infection of 25 million oocysts E. acervulina to assess the response of immune and non-immune older birds raised on 2 dietary levels of vitamin A. The term "immune" is used to denote a primary infection at an earlier date, and does not indicate any particular degree of immunity.

1. Primary Infection

The 2 groups receiving a primary infection with E. acervulina were D, low vitamin A diet, and H, normal vitamin A diet. Both groups of birds showed a severe reaction to the infection. All birds had diarrhea by day 3, and the feces were almost completely liquid with evidence of sloughed epithelium and bile present. By day 4 the birds of group D showed complete anorexia and adipsia but the birds of group H were not so severely affected. In both groups the birds were very depressed with congested or cyanotic combs and wattles, and pale, dry shanks. By day 6 the birds on normal vitamin A levels began to recover; the anorexia lessened and the feces, although scant and mucoid, were less liquid. By day 8 the birds of this group looked normal, and by day 9 their appetites had completely returned and the feces were back to normal. The birds on the low dietary vitamin A levels, group D, had a much stronger reaction to the infection.

The depression and diarrhea were more marked, and 4 out of the 6 birds died from the disease. The first death was on day 9, and this bird had lost 25 percent of its body weight compared to its inoculation weight. Bird 2 died on day 11 having lost 35 percent body weight; bird 3 on day 13 with a 40 percent loss in body weight; and bird 4 on day 17 with a 48 percent loss in weight. Post mortem examination of the dead birds revealed extreme emaciation and dehydration in all of them, plus a nephritis characterised by pale, mottled kidneys containing heavy urate deposits. All birds showed some evidence of intestinal damage, mostly involving the duodenum. Bird 1 had the most severe pathology with areas of hemorrhage extending to the duodenal-jejunum junction. The other 3 birds did not show such severe damage but all revealed evidence of epithelial sloughing and the walls of the intestine were thinner than usual. The birds of group D were moribund during most of the experimental period, and no evidence of normal feces was seen until day 12 in the surviving birds. Anorexia was complete, in all birds that died, from day 3 until their time of death.

Table XI shows the oocyst production of the 4 groups involved in this 3rd experiment. The group D total is probably lower than could be expected if there had been a higher rate of survival. This low vitamin A group had its peak production on

day 5, while the normal vitamin A group, H, had a peak oocyst production on day 4 followed by an abrupt drop on day 5. It is interesting to note that in the primary infection of all normal dietary vitamin A groups examined, the peak production is followed the next day by a drop of almost exactly 50 percent.

Table XII gives the total average weights of all the groups throughout the experiment. It can be seen that both the low vitamin A groups, D and B, were below the weights of groups F and H at the start of the experiment, but the differences were not statistically significant due to the high standard deviation present in all groups. However, by day 8, group D was significantly lower ($P \leq .01$) in weight than group H, and by day 14 the survivors of group D retained this difference at the 5 percent probability level. By day 14 the surviving birds of group D had lost about 20 percent of their starting body weights while the birds on higher levels of vitamin A (G) had lost only 2½ percent of their weight. The weight changes which took place during the course of the experiment are examined in Table XIII. Both groups receiving a primary infection showed a continual weight loss up to day 8. Coinciding with their regained appetites, the birds of group H showed a dramatic recovery of their lost weight between days 8 and 10, when they gained an average of 118 grams in the 2 days. The

survivors of the dessimated D group actually began to show a weight gain between days 10-12, but the continuing losses of the moribund birds maintained the group losses shown on Table XIII.

2. Secondary Infection

The low dietary vitamin A group (B) and the normal vitamin A group (F) were both given a primary infection at 5 weeks old in Experiment 1. This secondary infection was given 14 weeks later to test the degree of immunity retained in both groups.

Unlike the 2 groups receiving their primary infection, these two immunized groups did not exhibit a marked clinical response to the infection. There was a slight scouring present on days 4-6, and some evidence of anorexia. The low vitamin A group (B) showed less appetite during this acute period than the normal vitamin A group (F), and the feces were more scanty. Also group B birds did not return to their pre-infection feed consumption by the end of the experimental period. One bird in group B began to show ataxia and leg paralysis at day 14, and was killed at day 16. No evidence of coccidiosis was found in the intestinal tract, but microscopic examination revealed an overwhelming mold population present.

The oocyst production for these 2 "immune" groups is shown in Table XI, and unlike the findings in Experiment 2, the low vitamin A birds had almost twice the oocyst production of

the normal group. This conformed more to the findings in a primary infection. However, it can be seen that some immunity was retained in both groups as the numbers of oocysts passed was far below that of groups D and H.

An examination of the total weights throughout the experiment, as shown in Table XII, demonstrates that group F was the only group to show an increase in weight during the first 14 days after inoculation. Group B was 47 grams lighter than its inoculation weight at day 14, a 2 percent loss. However, by day 21 the 2 groups had the same relative weights as they had at day 0. When the weight changes are analysed in Table XIII, it can be seen that both the immune groups lost weight during the first 6 days of the infection, and group B lost more than twice that of group F. The reason for the weight loss in group B between day 10-14 is partially explained by the failing weight of the bird which was subsequently killed.

It was demonstrated in Experiment 2 that a primary infection with E. acervulina gave marked protection against a secondary infection 4 weeks later. From this present experiment it can be seen that this protective immunity is retained, in a lowered degree, 14 weeks after the primary infection. Unlike the results of Experiment 2, the low dietary vitamin A group did not show a higher degree of immunity than the birds on a

normal vitamin A diet. The average weight losses, and the oocyst counts, were both higher in group B than in Group F. However, this experiment confirmed the findings of Experiment 2 that the degree of protection afforded by the immunity is much more obvious in the low vitamin A group. Although the normal vitamin A group (H) exhibited a severe weight loss in the acute stages of the disease, this loss was regained so rapidly that in 14 days after inoculation the group H weight was equal to the immunized low vitamin A group (B), and not significantly lower than the immune normal vitamin A group (F). On the other hand, a comparison of groups B and D during this period shows a significant difference in weights ($P < .01$) plus a high mortality rate in group D. As illustrated in Figure 6, the normal vitamin A groups show only a 2 percent difference in weight between the immune and non-immune groups, but this difference rises to 18 percent in the low vitamin A group.

TABLE XI

AVERAGE DAILY OOCYST PRODUCTION IN MILLIONS IN 19-WEEK-OLD CHICKENS ON 2 DIETARY LEVELS OF VITAMIN A, AND 2 LEVELS OF IMMUNITY, AFTER INFECTION WITH 25 MILLION OOCYSTS OF E. ACERVULINA PER BIRD

Day	Low vitamin A ration		Normal vitamin A ration	
	Immune (B)	Non-immune (D)	Immune (F)	Non-immune (H)
4	3	40	8	135
5	40	220	30	60
6	40	60	10	30
7	25	50	20	30
8	6	55	6	40
9	20	25 (5)*	6	20
10	10	20 (5)	7	10
12	0.1	2 (4)	3	4
14	0.03	2 (4)	0.08	1
Total oocysts per bird	145	474	90	330
Reproductive Index	6	19	4	13

* number of surviving birds out of 6.

TABLE XII

AVERAGE WEIGHT IN GRAMS OF 19-WEEK-OLD CHICKENS, ON 2 DIETARY LEVELS OF VITAMIN A, AND 2 LEVELS OF IMMUNITY, FOLLOWING INFECTION WITH 25 MILLION OOCYSTS OF E. ACERVULINA

Day	Low vitamin A		Normal vitamin A	
	Immune (B)	Non-immune (D)	Immune (F)	Non-immune (H)
0	1785	1763	1848	1911
4	1737	1668	1823	1826
6	1697	1541	1808	1728
8	1726	1394	1866	1707
10	1754	1294(5)*	1902	1825
12	1752	1280(4)	1912	1848
14	1738	1417(3)	1911	1866
21	1889	1620(2)	1945	1922

* number of surviving birds out of 6.

TABLE XIII

DAILY MEAN WEIGHT CHANGES IN GRAMS IN IMMUNE, AND NON-IMMUNE 19-WEEK-OLD CHICKENS ON 2 DIETARY LEVELS OF VITAMIN A, FOLLOWING INFECTION WITH 25 MILLION OOCYSTS OF E. ACERVULINA

Between days	Low vitamin A		Normal vitamin A	
	Immune (B)	Non-immune (D)	Immune (E)	Non-immune (H)
0-4	-48	-95	-25	-85
4-6	-40	-127	-15	-98
6-8	+29	-147	+58	-21
9-10	+28	-100 (5) *	+36	+118
10-12	- 2	-2 (4)	+10	+24
12-14	-14	+137 (3)	-1	+17

* number of surviving birds out of 6.

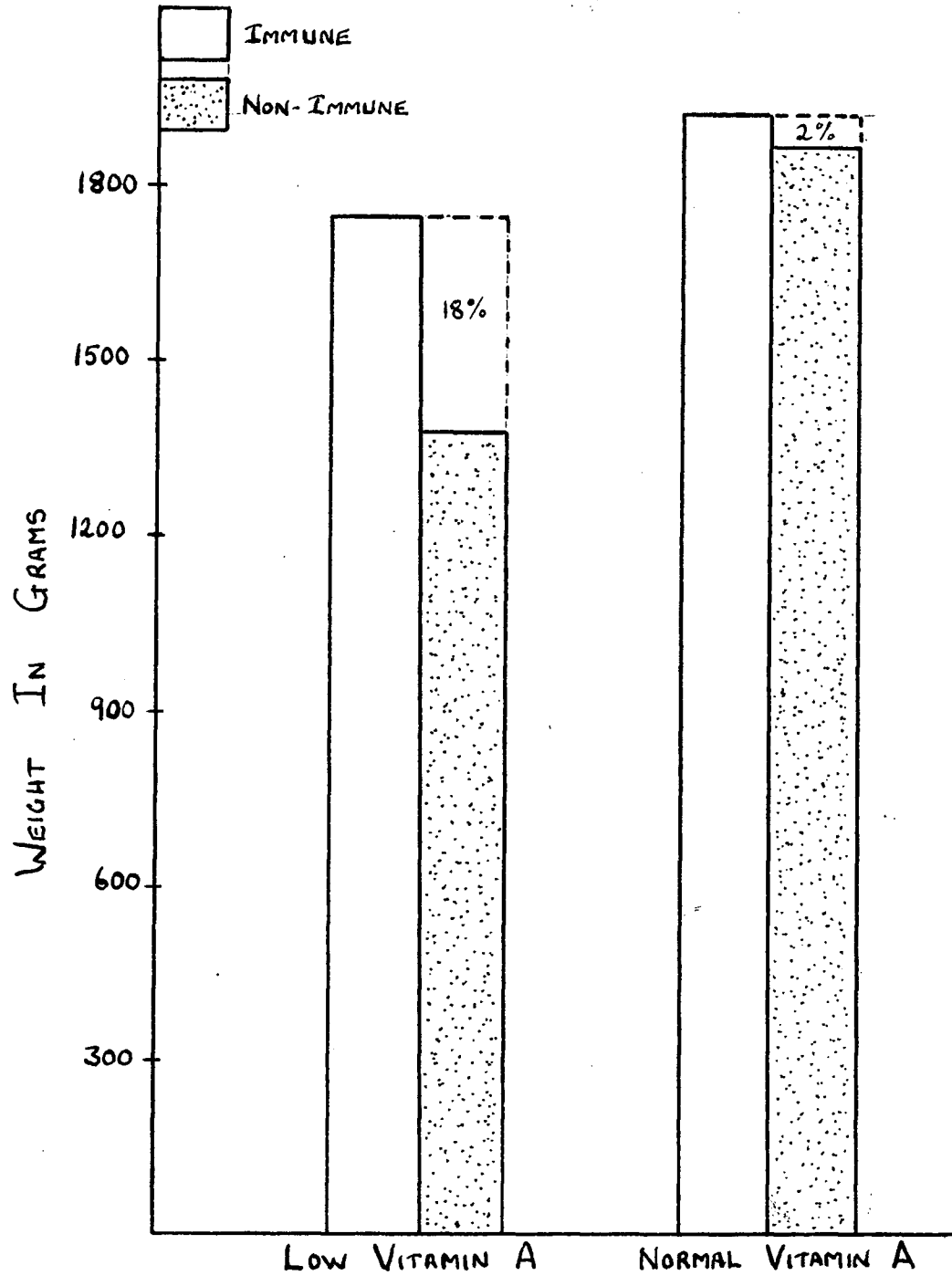


Figure 6. The effect of dietary vitamin A level on the mean weight of 19-week-old immune and non-immune birds 14 days after infection with E. acervulina

3. Post Mortem and Histological Examinations

Two birds from each group were killed on day 4 following inoculation, and the intestines checked for pathological lesions. Sections from each of the birds were examined to determine the extent of the parasitic invasion and development.

Post mortem findings:

Group B - Low vitamin A diet - immune

One of the birds showed a 3.5 percent weight loss at 4 days and examination of the intestine showed some sloughing of the epithelium in the duodenum, and areas of enteritis at the beginning of the jejunum. The second bird showed no clinical or post mortem evidence of infection, and had not lost weight.

Group D - Low vitamin A diet - primary infection

Both birds killed showed clinical signs of the disease and both had a 7 percent weight loss at day 4. Bird No. 1 demonstrated only some sloughing of the intestinal epithelium on post mortem examination, but bird No. 2, which had a more severe diarrhea, showed pronounced lesions in the intestine. The entire intestine anterior to the yolk stalk revealed a spotty enteritis.

Group F - Normal vitamin A - immune

Bird No. 1 showed no clinical or post mortem signs of coccidiosis. The second bird had a weight loss of less than

1 percent but exhibited severe diarrhea. However, there was no evidence of enteritis on post mortem examination, although the duodenum and jejunum both showed severe sloughing of the epithelium.

Group H - Normal vitamin A - primary infection

Both the birds had severe diarrhea and depression, and showed a 5 percent loss in weight since inoculation. Post mortem examination showed that bird No. 1 had severe sloughing of the entire anterior portion of the intestinal mucosa, and the jejunum posterior to the duodenum was inflammed. The second bird showed less pathology, but severe epithelial sloughing was evident.

Histological examinations:

Almost all the sections revealed evidence of severe sloughing of the villi epithelium so that in many cases the lamina propria was completely unprotected. There was also a great deal of edema evident in the villi of all groups.

The difference between slight and severe pathology in the intestine appeared to be the degree of involvement. In the severe cases, a far greater number of cells were invaded with few areas in the sections being free of the parasites. The crypts and neighbouring villi were packed with trophozoites and schizonts of different sizes and stages of maturity. The gametocytes and oocysts present were not confined to the tips

of the villi as reported from various sources, but were found also in the crypts. In the intestines showing only sloughing of the epithelium, there were far fewer areas invaded, and the affected regions did not show the same number of cells affected. Both the low vitamin A groups revealed a more extensive involvement than the groups on normal vitamin A diets. The non-immune group D showed the most extensive invasion of the intestinal cells and also proportionately more oocysts in its sections than any other group.

V. DISCUSSION

The results of this investigation demonstrate that a diet containing only 10 percent of the recommended level of vitamin A does not necessarily affect the weight gains or clinical health of the birds receiving it. However, if these same birds are subjected to coccidiosis, the effects of this suboptimal vitamin A become immediately apparent. In these experiments, infection with E. acervulina was more severe in all groups on the low vitamin A diet, as determined by a comparison of the oocyst production, weight gains, and mortality between birds on low dietary levels of vitamin A and normal levels.

A. Oocyst Production

Brackett and Bliznick (1952b) have reported that the number of oocysts produced by a coccidial infection are affected by at least 6 factors.

1) The inherent potential of the parasite in a non-immune host.
2) The immunity or resistance developed by the host.
3) The "crowding effect".
4) Competition with other coccidial species or infective agents.
5) Nutrition of the host.

6) Strain differences of the host.

The reproductive index, or potential, of a coccidium is the maximum number of oocysts produced per oocyst inoculated. It depends on the number of schizogonous generations and the numbers of merozoites produced in each generation. With E. acervulina, the theoretical potential is $\frac{8 \times 20 \times 16 \times 10 \times 36}{2}$ = 455,000 per oocyst injected, if the endogenous stages reported by Vetterling and Doran (1966) are correct. Hein (1968a) demonstrated that the reproductive potential dropped with increasing inoculum, which confirms findings by other authors and the results from these experiments. Warren and Ball (1967) reported the highest R.I. (100,000) of any investigation. This resulted from an inoculum of 500 oocysts in 3-week-old chicks. Table XIV gives a list of reproductive indexes obtained by various authors and demonstrates the decrease in R.I. with increasing inoculum. The results of this investigation are also included.

As first demonstrated by Brackett and Bliznick (1952b) E. acervulina is characterized by an extremely high oocyst production. Although its reproductive potential is lower than that of E. brunetti and E. tenella, its oocyst potential is far higher than any other avian species of coccidia. Tyzzer et al (1932), and Brackett and Bliznick (1952b), have demonstrated

TABLE XIV

THE REPRODUCTIVE INDEX OF *E. ACERVULINA* FOLLOWING INCREASINGLY LARGER INFECTIVE DOSES AS REPORTED BY VARIOUS AUTHORS

Inoculation - number of oocysts per bird	R.I.	Age of birds	Author
50	72,000	unknown	Brackett and Bliznick (1952b)
500	100,000	3 weeks	Warren and Ball (1967)
900	38,000	7 weeks	Long (1967)
1250	43,000	6 weeks	Hein (1968a)
320,000	1,800	6 weeks	Hein (1968a)
500,000	1,026	3 weeks	Rose and Long (1962)
1 million	302	6 weeks	Hein (1968a)
3 million - normal vitamin A group	120	5 weeks	Coles
3 million - low vitamin A group	205	5 weeks	Coles
5 million	44	6 weeks	Hein (1968a)
10 million - normal vitamin A group	43	9 weeks	Coles
10 million - low vitamin A group	86	9 weeks	Coles
20 million	8	6 weeks	Hein (1968a)
25 million - normal vitamin A group	13	19 weeks	Coles
25 million - low vitamin A group	19	19 weeks	Coles

that the highest yields of oocysts are obtained with light infections in the case of E. tenella, E. brunetti, E. maxima and E. necatrix, and with high inoculums the oocyst production decreased. However, these authors found that E. acervulina did not show this correlation, and instead revealed an almost unlimited oocyst production capacity. It would appear that this finding is related to a lower immune response to this species, and if this is the case the higher oocyst production shown by the groups on a ration low in vitamin A may be due to a suppression of the immunological mechanisms in the deficient host.

It has been reported by several authors that age is a factor in oocyst production; the older the birds, the greater the potential production. Hein (1968a) demonstrated that an infective dose of 5 million oocysts of E. acervulina in 2-week-old chicks gave a total production of 37 million, while the same inoculum in 6-week-old chicks gave a total of 225 million. Krassner (1963) reported that the mean oocyst production increases proportionately with host age, and attributed this finding to the fact that more intestinal cells are available for parasite penetration in older birds. In this present investigation the maximum oocyst production occurred in 9-week-old birds following an infective dose of 10 million

oocysts. The low vitamin A group had a total oocyst production of 860 million, twice the production of the birds on normal levels of vitamin A. This doubling of oocyst production under conditions of low dietary vitamin A was seen also in the 5-week-old birds, but in the 19-week-old birds the mortality was so high that an accurate assessment was difficult to make. Figure 7 compares the oocyst production in the 3 experiments. Ackert et al (1931) suggested that the greater parasitism with nematodes in birds on low vitamin A diets was due to reduced peristalsis. If this is correct, increased oocyst production may result from this same phenomena, as a slower peristalsis would allow a greater number of sporozoites more time to invade the cells.

The nutritional requirements of the coccidium can be involved in total oocyst production. Warren (1968) has investigated the vitamin requirements of E. acervulina and has found that thiamine, riboflavin, biotin and nicotinic acid are essential for optimum oocyst development and production. However, he reports that a diet deficient in vitamin A resulted in an oocyst production twice that of the normal controls. Britton, Hill and Barber (1964) have demonstrated that low protein levels in the diet cause reduced oocyst production because a low trypsin secretion results in a decreased excystation.

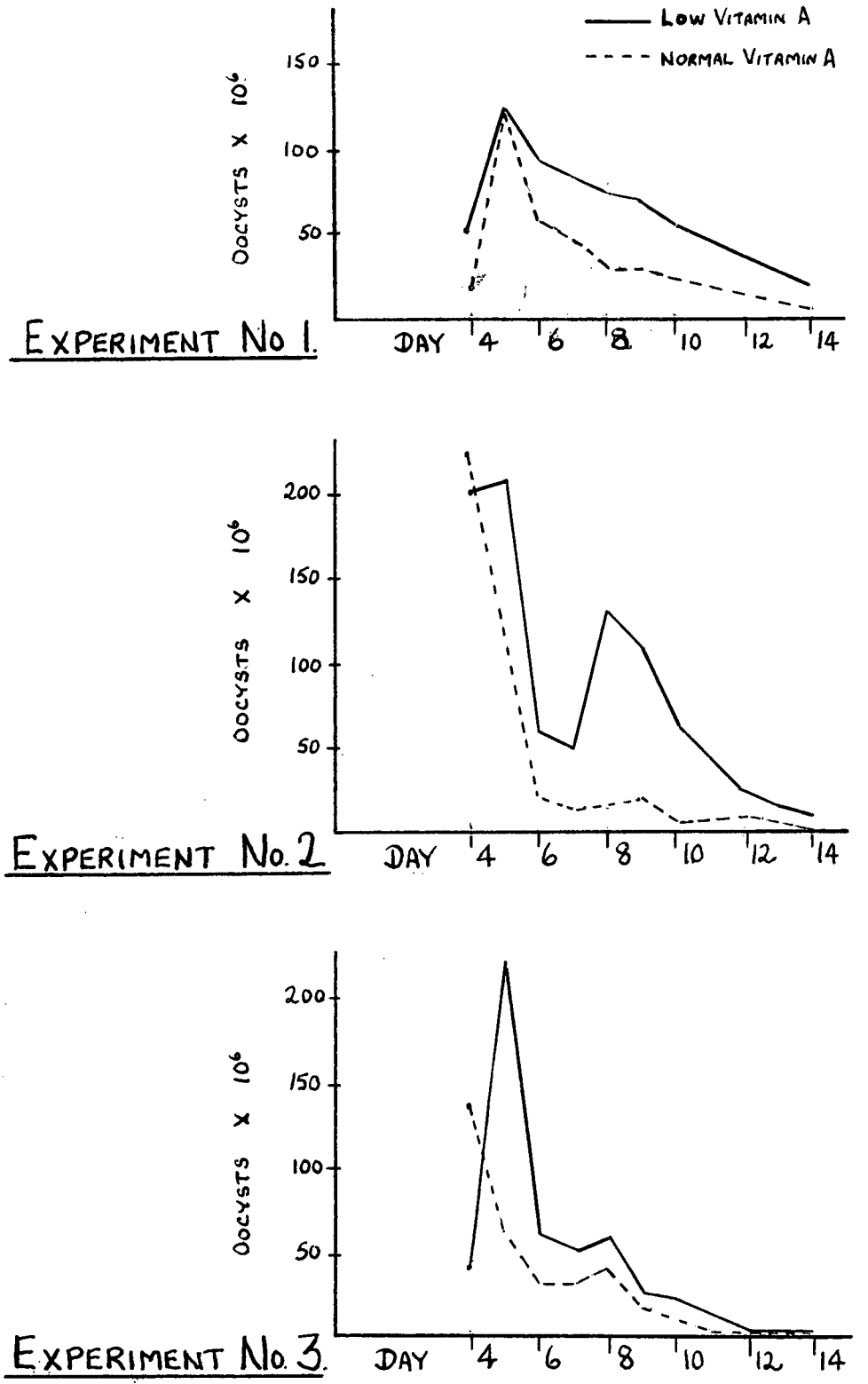


Figure 7. A comparison of the daily oocyst production in chicks on Experiments 1, 2, and 3

Long (1968a) reported that the breed and strain of the bird plays a significant part in its susceptibility to a coccidial infection as demonstrated by the oocyst counts. He found that White Leghorns are more susceptible than Rhode Island Reds.

B. Weight Gains

In the 3 experiments comprising this study one of the main findings was a much reduced weight gain following infection in the birds on low levels of vitamin A. In Experiment 1, using 5-week-old chicks and an inoculum of 3 million oocysts, the low vitamin A group had a 2 week weight gain amounting to only 37 percent of that shown by its non-infected control, while the normal vitamin A infected group had a gain equal to 70 percent of its control. In Experiment 2, these figures were 26 percent and 80 percent respectively. These findings concur with those of Erasmus, Scott, and Levine (1960) and Waldroup et al (1963). The weight loss was due mainly to the anorexia characterizing the acute stage of the infection. Reed and Pitois (1965) reported that from day 4-6 following an infection with E. acervulina, feed and water intakes are reduced at least 50 percent. Preston-Mafham and Sykes (1967b), using equalized feedings between infected and control birds, found that with a E. necatrix infection 70 percent of the weight loss was due to

a reduction in feed intake, and 30 percent to impaired absorption in the intestine. Erasmus et al (1960), using the same techniques, reported similar findings in birds on 2 levels of vitamin A subjected to a mixed infection of E. acervulina and E. tenella. An analysis of their results show that the low vitamin A group was 9 percent lower in weight than its non-infected control, while the high vitamin A group was only 4 percent lower than its control. This difference is probably due to a decreased absorption in the lower vitamin A group. Long (1968), and Preston-Mafham and Sykes (1967b) reported that an infection with E. acervulina resulted in a loss in serum proteins into the gut lumen, as demonstrated by the Pontamine sky blue dye test. This loss is at a maximum 3-5 days after infection, coinciding with the period of depressed absorption, and is indicative of a change in gut permeability. Long also reported changes in permeability 3½-7 hours after inoculation which he attributed to the sporozoite entry into epithelial cells.

In the experiments of this investigation, it was noted that the feed and water intake of the birds during the acute stage was practically nil. The anorexia was more pronounced in the low vitamin A groups in all the 3 experiments. As these experiments did not equalize feed intake between the infected

and non-infected controls, and as the low vitamin A infected groups showed a greater degree of anorexia, it would follow that the main weight differences between the groups were due to this greater loss of appetite.

C. Mortality

Mortality is not a characteristic finding in an infection with E. acervulina. This is especially true when the birds are subjected to only one infective dose as demonstrated by Dickinson and Scofield (1939) and Hein (1968a). Morehouse and McGuire (1956) reported 6 percent mortality in chicks after a single 5 million oocyst inoculation, but this is unusual and could be due to a particularly virulent strain of the organism. In the 3 experiments reported in this paper, the highest mortality appeared in 19-week-old, non-immune birds on the low level of dietary vitamin A. Four of the 6 birds in this group died, and death was associated with a weight loss of over 30 percent of the total body weight. This weight loss was due primarily to the severe anorexia and adipsia displayed by the infected birds on the low vitamin A diet. Age may have been a factor which aggravated the effects of the infection in experiment No. 3. Tyzzer et al (1932) reported that older birds were more susceptible to infection with E. necatrix and this same may be true with E. acervulina. Brackett and Bliznick (1952a), also

working with E. necatrix, demonstrated that with equal oocyst ingestion the younger birds were more susceptible, but when the dose was calculated to give equal numbers of oocysts per gram of body weight, the older birds were the more severely affected.

D. Immunity

As demonstrated by the higher oocyst production and reproductive index in birds on the low vitamin level in the diet, there was a delay in the appearance of the immune response. The reason for this delay may be associated with a reduced adrenal cortical function under low vitamin A conditions as reported by Glick (1963). It was earlier demonstrated by Challey (1962) that in birds affected with E. tenella there is an increase in the secretion of adrenal corticosterones, and he attributed this increase to the stress of the disease and the increased sythesis occurring in the body as a result. Glick (1963) found that the reduction of adrenal cortical hormones could be determined by a lower heterophil count in the circulation following an injection of ACTH. Newcomer (1957) has suggested that increased heterophils are a measure of the response of birds to acute stress. The appearance of numerous heterophils in the lamina propria of birds 3 hours after an inoculation with E. acervulina oocysts, would agree with this suggestion. The fact

that 2 of the 3 birds on low vitamin A levels did not show the large number of heterophils observed in the other groups, may correlate with the report of reduced adrenal cortical secretions under low vitamin A conditions.

The strength of the immunity, once established, was not related to the level of vitamin A in the diet, but rather to the severity of the initial infection. This finding confirms earlier reports by Tyzzer (1929) and later findings by Hein (1968b) that the degree of immunity to coccidiosis is directly related to the severity of the infection. The nature of this immunity has been widely investigated, and it is generally considered that a local factor is primarily responsible for the protection. Rose (1963) suggests that a sensitivity of the intestinal epithelium results from the presence of circulating antibodies, and this sensitivity is responsible for the inhibition of schizont development. The effects of immunity are demonstrated in the sections taken 48 hours after the start of infection. The immune birds, regardless of the levels of vitamin A fed, showed an inhibition of parasite development. This effect of immunity on endogenous forms confirms the findings of Long, Rose, and Pierce (1963), Horton-Smith and Long (1963) and Leathem and Burns (1967). All these investigators found that there was an invasion of the villi by the sporozoite, and a migration to the

crypt. However, the sporozoites either failed to develop further, or else a trophozoite developed which did not mature into a schizont.

The birds on the lower dietary level of vitamin A did not retain their immunity as well as the birds on normal diets, but the difference was not significant. However, the fact that there was considerable protection still effected by both groups 100 days after a primary infection, is very interesting as most investigators report a shorter period of protective immunity with E. acervulina. However an immunization dose as high as 3 million oocysts has not been reported, and it would appear that with E. acervulina an inoculum of this size must be used to establish effective immunity.

VI. SUMMARY AND CONCLUSIONS

1. Experiments were conducted to investigate the pathological effects of an infection with E. acervulina in birds fed a diet containing 440 I.U. vitamin A per kilogram, compared to birds fed a similar diet containing 4400 I.U. vitamin A per kilogram.

2. No symptoms of deficiency were observed in the low vitamin A groups, and no significant weight differences were recorded between the non-infected normal vitamin A and low vitamin A groups.

3. The effect of the low level of vitamin A in the diet was not manifested until the birds were infected with E. acervulina.

4. Following an infection with E. acervulina, the birds on the low level of vitamin A showed significantly lower weight gains, and higher mortality and oocyst production, than did the birds receiving the higher level of vitamin A.

5. Birds fed the low level of vitamin A demonstrated a slower development of the immune response to E. acervulina infection.

6. The degree of immunity to E. acervulina was related to the severity of the initial infection.

7. The low level of vitamin A used in these experiments did not result in a loss of epithelial integrity in the intestine.

8. There were no detectable effects of low vitamin A dietary levels on the endogenous stages of the parasite. The sporozoite invasion of the intestinal epithelium appeared to be similar in all groups. The inhibitory effect of immunity on the development of the first generation schizont was also similar in birds on both dietary levels of vitamin A.

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