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**Davis, Catherine Young**

IMMUNOGLOBULIN CONCENTRATIONS IN SERUM AND SECRETIONS OF VITAMIN A-DEFICIENT BROILER CHICKS FOLLOWING NEWCASTLE DISEASE VIRUS VACCINATION

*Iowa State University*                                    PH.D.   1985

Immunoglobulin concentrations in serum and

secretions of vitamin A-deficient broiler chicks following

Newcastle disease virus vaccination

by

Catherine Young Davis

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# INTRODUCTION

If one were to quickly review our world's history of famine and pestilence, even today, we could easily find a link between various forms of malnutrition, often severe marasmus or kwashiorkor, and increased susceptibility to infectious diseases. It would be ideal if one could quantitate the effect of the deficiency of each essential and nonessential nutrient on each distinct component of host defense. Within the past two decades, a large amount of scientific research has been conducted to determine relationships between specific nutrients and immune functions. One such nutrient, vitamin A, has received considerable attention recently. Numerous studies have demonstrated that vitamin A deficiency, in humans and animals, greatly increases their susceptibility to infection. The studies of immune function in vitamin A deficient states have largely concentrated on an analysis of systemic immune responses and relatively little attention has been given to the local immune apparatus which operates at specialized sites in the body such as the respiratory and intestinal tracts. In humans and animals suffering from vitamin A deficiency, infections at these sites make a major contribution to morbidity (Chandra and Newberne, 1977). Vitamin A functions in maintaining anatomical barriers of the body, suggesting that the primary nonspecific host defense in which vitamin A plays an essential role is the resistance of mucous membranes to microbial colonization and/or absorption of potentially dangerous antigens. Once microorganisms have penetrated these first lines of defense, vitamin A may then play a secondary role in systemic immune response.

The present study examines the concentration of immunoglobulins in serum and secretions from selected tissues of vitamin A deficient chicks after vaccination with Newcastle disease virus. The ability of vitamin A deficient chicks to produce specific serum antibody to Newcastle disease virus was also determined. A comparison of two sources of vitamin A, retinoic acid and retinol, in maintaining host immune defense was made.

## LITERATURE REVIEW

### Susceptibility to Infection

As early as 1927, Sherman and Burtis (1927-28) observed that vitamin A deficiency in rats greatly increased their susceptibility to infection. Since then, over 50 scientific papers have described diseases of bacterial, viral, or protozoal origin in which vitamin A deficiency results in greater frequency, shorter survival periods and a much greater mortality rate than controls. The increase in susceptibility to infection is due at least in part to the presence of greater numbers of infective agents residing within the vitamin A deficient animal. Observations of vitamin A deficient rats infected with a malarial parasite indicated that high mortality was the result of a greater number (60-90%) of red blood cells carrying the parasite after infection. In comparison, pair-fed, vitamin A supplemented rats had a lower mortality and only 8 to 45% of their red blood cells carried the parasite. After oral supplementation of retinyl acetate, the vitamin A deficient rats were able to recover from the malarial infection (Krishnan et al., 1976). Similarly, Cohen and Elin (1974) found that five hours after challenge with either a bacterium or fungus, mice pretreated with 3000 I.U. of vitamin A had sterile blood, whereas the control animals showed persistent bacteremia until death. Tengerdy and Brown (1977) noticed that oral administration of vitamin A to growing broiler chicks reduced mortality resulting from E. Coli infection.

Increased susceptibility to infection is due in part to a reduced local resistance at the site of entry by infective agents. Darip et al. (1979) have shown that an oral dose of Angiostrongylus cantonensis, when

given to vitamin A deficient rats, results in greater severity of illness and less immunity gained against subsequent reinfection than when the same dose is given to control rats. The infective agent was able to penetrate the intestinal mucosa of vitamin A deficient rats more effectively as determined by a greater number of worms recovered from the pulmonary arteries and lungs in deficient rats as compared with controls. A reduction in the ability to prevent penetration by infective agents into epithelial tissue is consistent with the fact that one of the functions of vitamin A is to maintain morphological and functional integrity of epithial tissues. However, when larvae were deposited directly into the tissue by a subcutaneous route, deficient rats still developed a more severe infection than controls.

In vitamin A adequate chickens, intranasally inoculated with Newcastle disease virus (NDV), the virus destroyed epithelium of the upper respiratory tract and was replaced by mucociliated cells. In vitamin A deficient chickens, similarly infected with NDV, the damaged cells were replaced by keratinized squamous cells (Bang and Bang, 1969). Additional studies with vitamin A deficient chickens revealed depletion of lymphocytes and plasma cells from nasal and paranasal lymphoepithelial tissues. Infection of the vitamin A deficient birds with NDV produced further depletion of plasma cells, subnormal inflammatory response, and keratinization of nasal mucociliated epithelia (Bang et al., 1972). These modifications of submucosal areas of the nasal fossa predisposed the vitamin A deficient chickens to infection with NDV. Bang and Foard (1971) observed 100 times more virus in throat swabs of vitamin A deficient

chickens than in those of controls after both groups were given NDV by intranasal instillation.

## Humoral Immunity During Vitamin A Deficiency

### Antibody production

Animals and humans reported to be in a state of vitamin A deficiency generally exhibit a decrease or no difference in antibody titer in response to a variety of antigens when compared with controls. A study which combined a vitamin A deficiency with concomitant protein-energy malnutrition showed that hemagglutinin titers of vitamin A deficient rats in response to sheep erythrocytes were diminished as compared with normally fed rats (Krishnan et al., 1974). However, the antibody response in the vitamin A deficient rats was not significantly less than that of pair-fed control rats. The decrease in antibody response may have been the result of decreased food intake associated with vitamin A deficiency and not the lack of a single dietary nutrient. More recently, Chandra and Au (1981) examined the plaque-forming cell response of vitamin A deficient rats after immunization with sheep erythrocytes. The number of spleen cells producing antibody against sheep erythrocytes was decreased in animals fed the vitamin A deficient diet as compared with pair-fed controls. Panda and Combs (1963) detected lower agglutinin responses to S. Pullorum challenge in chicks fed diets partly deficient in vitamin A as compared with controls. The partial deficiency of vitamin A interfered significantly with optimum agglutinin response, yet normal growth of the chicks was maintained.

Contrary to these studies, others have observed that antibody production was not influenced by vitamin A status. Normal children and children with ocular signs of vitamin A deficiency were vaccinated with diptheria and tetanus. After immunization, there was an increase in antibody titer in all the children. There was, however, no significant difference between the two groups (Kutty et al., 1981). Similarly, Underdahl and Young (1956) found that hemagglutination inhibition titers in vitamin A deficient mice infected with swine influenza equalled those of control mice.

## Vitamin A as an adjuvant

An adjuvant-like effect to antibody production has been noted in animals treated with various doses of vitamin A before or after immunization with antigens. Intraperitoneal injections of vitamin A five days preceding or immediately after sensitization with sheep red blood cells led to a large increase in the production of hemagglutinin antibodies by mice (Jurin and Tannock, 1972). Pretreatment with doses no less than 1000 I.U. and no greater than 3000 I.U. of vitamin A daily for four consecutive days before immunization with sheep red blood cells increased the number of antibody-forming cells generated in the spleen of mice. Antibody response to immunization with the hapten protein conjugate, dinitrophenol-ovalbumin, also was enhanced by vitamin A pretreatment (Cohen and Cohen, 1973). Leutskaya and Fais (1977) found that chicks given a normal diet until immunization with human serum albumin and then fed a vitamin A deficient diet until 7, 14, and 21 days postimmunization exhibited serum antibody concentrations that were 20 to 50% lower than those of birds fed

a normal diet throughout the experiment. The differences between the two groups were most pronounced on the seventh day following immunization. A single peroral dose of 30,000 I.U. of retinyl palmitate any day after reimmunization, but most notably after the third day, increased the antibody content of serum of normally fed chicks.

Contrary to these animal studies, observations made in children indicate that antibody production is not influenced by vitamin A supplementation. Treatment of deficient children with vitamin A failed to increase tetanus antitioxin levels as compared with control groups similarly immunized with tetanus toxoid (Brown et al., 1980). Subsequent studies with mice, however, demonstrated that the animals required unusually high doses of vitamin A before favorable antitoxin responses were observed. It was concluded that the relatively large doses of vitamin A needed to produce this effect in mice would probably cause undesirable side effects if administered to children.

The local as well as systemic antibody response has been shown to be enhanced by vitamin A treatment. Sirisinha et al. (1980) fed rats, previously depleted of hepatic vitamin A reserves, diets supplemented with retinoic acid. Rats that were given a split dose of retinyl palmitate 1 and 2 days before retinoic acid was ultimately removed from their diet had greater anti-dinitrophenol activities in their intestinal fluid after oral immunization with dinitrophenol 50-bovine gamma globulin than did rats pretreated with retinyl palmitate.

Oral doses of vitamin A given two consecutive days before and on the day of intragastric administration of bovine serum albumin (BSA) increased

the production of antibody to this protein in mucosal tissue as compared with animals given BSA alone (Falchuk et al., 1977). In the same study, very low and very high doses of BSA, in the absence of vitamin A, induced tolerance to this protein. In the presence of vitamin A, these doses of BSA stimulated production of antibodies.

The mode of action of vitamin A as an adjuvant has not been elucidated. Evidence indicates that this activity of vitamin A may be the results of its effect on T cells. In neonatal thymectomized mice depleted of T cells by administration of rabbit-anti-mouse lymphocyte serum, the adjuvant effect of retinol to BSA observed in normal mice was decreased. T cells, therefore, may be necessary for the mechanism of action of retinol as an adjuvant. Vitamin A has been shown to stimulate blast transformation and proliferation of T cells in thymus-dependent areas of draining lymph nodes (Taub et al., 1970).

## Immunoglobulin production

Despite extensive reports on the antibody response of vitamin A deficient animals and humans, few studies have been conducted which examine the effect of vitamin A deficiency on immunoglobulin production. In children with Down's Syndrome, the plasma concentration of vitamin A was reduced. Palmer (1978) investigated suboptimal vitamin A status as a probable cause for the greater frequency of respiratory and gastro-intestinal infection observed in children with Down's Syndrome. The increased susceptibility to infection by Down's patients was also accompanied by greater serum IgG concentrations and decreased IgM concentrations as compared with normal controls. The occurrence of increased serum

IgG in children with Down's Syndrome compared with normal children may reflect an increased response due to continuous antigenic stimulus from viral infections. After treating Down's Syndrome children with oral doses of vitamin A, plasma vitamin A concentrations increased. There was a significant decrease in the incidence of infection and a corresponding decrease in serum IgG concentration. Reduction in the frequency of infection and serum IgG concentrations after treatment with vitamin A suggests protection by epithelial linings was improved.

Results from numerous publications associating vitamin A deficiency with reduced immune function led Gershwin et al. (1984) to test the hypothesis that a reduction in autoantibody production may be mediated by vitamin A deficiency. However, when they fed New Zealand Black mice, susceptible to autoimmune disease, a vitamin A-free diet, the vitamin A deficiency accelerated the progression of autoimmune disease compared with pair-fed controls. Those mice fed a vitamin A-free diet for a 6-month period had significantly greater serum IgG and IgM concentrations than pair-fed control mice. There was no significant difference in serum IgA concentrations.

Serum IgG and IgA concentrations did not change during a period in which retinoic acid (RA) was supplemented and then withdrawn from the diet of rats depleted of hepatic vitamin A reserves (Sirisinha et al., 1980). Similarly, children with ocular signs of vitamin A deficiency and normal children had similar concentrations of plasma IgG, IgM, and IgA. The concentrations of plasma immunoglobulins were similar between the two

groups of children before and 14 days after immunization with diptheria and tetanus vaccine (Kutty et al., 1981).

Generally, in contrast to normal or elevated serum immunoglobulins, secretory immunoglobulins in vitamin A deficient animals and humans are decreased. For example, Sirisinha et al. (1980) found secretory IgA (SIgA) concentrations to be 180.2 ug/ml of intestinal fluid in rats depleted of hepatic vitamin A and maintained on diets supplemented with RA. When RA was removed from the diet for a 10-day period, SIgA concentration in intestinal fluid fell to 134 ug/ml. Intestinal fluid IgG was not affected by removal of vitamin A from the diet. Palmer (1978), however, found higher concentrations of IgA in the saliva of children with Down's Syndrome. Down's Syndrome is characterized by reduced plasma vitamin A concentrations. The occurrence of increased SIgA as compared with normal controls may reflect an increased response due to a greater frequency of respiratory and gastro-intestinal infection observed in these children.

Impaired secretory immunity may contribute to the increased frequency of infection often seen in association with vitamin A deficiency. Impaired SIgA production in vitamin A deficiency may be due to several factors. Studies by McDermott et al. (1982) which examined the intestinal localization of mesenteric lymphoblasts of vitamin A deficient rats provides evidence for one possible mechanism for reduced SIgA concentrations. In this study, lymphoblasts isolated from intestinal lymph nodes of vitamin A deficient and control rats were labeled with $^{125}I$ deoxyuridine and injected into recipient animals. A smaller proportion of

cells obtained from vitamin A deficient rats localized within the gut of recipient rats as compared with cells derived from donor vitamin A control rats. The difference occurred irrespective of the nutritional status of the recipient. Selective localization of mesenteric lymph node cells into the gut may require specific recognition of these cells by intestinal cells. Specific recognition may involve cell surface molecules which act as receptors. Vitamin A has been shown to affect the synthesis of specific glycoproteins found on the surface of cell membranes. Synthesis of receptor glycoproteins may be impaired during vitamin A deficiency. Therefore, localization of lymphoblasts responsible for making IgA antibodies in mucosal tissues would also be impaired.

Impaired SIgA production may also be the result of a reduction in the synthesis of secretory component by epithelial cells of vitamin A deficient animals. Segments of small intestine from deficient and control rats were examined by immunofluorescence for the presence of secretory component. A reduced staining for intracellular secretory component in the epithelial cells of vitamin A deficient rats suggested that the synthesis of secretory component was adversely affected by vitamin A deficiency (Sirisinha et al., 1980).

Circulating Lymphocytes During Vitamin A Deficiency

Nauss et al. (1979) found a decrease in the number of circulating lymphocytes and an increase in the number of circulating neutrophils as a consequence of early vitamin A deficiency in rats. Further studies indicated that the proportion of different types of lymphocytes were altered during vitamin A deficiency. Studies of children showing signs of

vitamin A, but not protein-energy malnutrition, showed that T lymphocyte concentrations in peripheral blood were lower than those of controls (Bhaskaram and Reddy, 1975; Kutty et al., 1981). The concentration of B lymphocytes in peripheral blood were not different between deficient and control children. Recently, Malkovsky et al. (1983) fed mice a conventional diet with or without supplementary vitamin A acetate. Fluorescence-activated cell sorter analysis revealed that vitamin A acetate-fed mice had a significantly higher proportion of Lyt 1.1 positive cells in their lymph nodes than control mice, whereas the percentage of Lyt 2.1 positive cells was unchanged. The action of vitamin A acetate in enhancing immunologic reactivity may be mediated by its ability to increase the proportion of helper T cells rather than suppressor or effector cell types.

Cell-Mediated Immune Response During Vitamin A Deficiency

In vitro mitogen assays of cellular immune function usually have shown that the rate of lymphocyte transformation is reduced during vitamin A deficiency, although normal or increased responses have been observed occasionally. Krishnan et al. (1974) found that the incorporation of ($^3$H-methyl) thymidine into DNA of cells, derived from thymus and spleen of vitamin A deprived protein-energy malnourished rats, was decreased as compared with that of control rats. Nauss et al. (1979) used the mitogens concanavalin A and phytohemagglutinin to stimulate T lymphocytes of vitamin deficient, control and pair-fed rats. The transformation response of splenic lymphocytes from vitamin A deficient animals was only one-third that of cells obtained from control or pair-fed animals. In contrast,

proliferative response of spleen cells obtained from vitamin A deficient New Zealand Black mice to phytohemagglutinin was greater than those from pair-fed and control mice (Gershwin et al., 1984). No difference in the proliferative response of spleen cells derived from vitamin A deficient New Zealand Black mice to the mitogen concanavalin A was observed. Similarly, Bhaskaram and Reddy (1975) found no difference in the phytohemagglutinin-stimulated rate of thymidine incorporation into cells of cultures of peripheral blood lymphocytes obtained from vitamin A deficient children as compared with control children.

Studies of proliferation responses have also involved the in vitro administration of retinoids to cultures of T cells derived from various tissues. The transformation response of T cells vary depending on the procedure employed. Retinol added to cultures of peripheral blood lymphocytes derived from healthy humans did not change the rate of lymphocyte proliferation in the presence or absence of phytohemagglutinin (Baranska et al., 1972). Similarly, Dennert and Lotan (1978) noted that various doses of RA added to cultures of normal mice spleen cells did not stimulate cell proliferation in the absence of a mitogen and, in the presence of the T-cell mitogens, phytohemagglutinin and concanavalin A, RA suppressed cell proliferation.

The inability to enhance proliferation of T cells by in vitro addition of vitamin A may be due to the absence of the appropriate factors in the culture media which are required to interact with the compound to modify the immune response. Furthermore, the entry rate of vitamin A into cells used in in vitro research may differ from that which occurs in whole

animals. In most research with isolated tissues or cells, vitamin is added to the medium in the free state. In whole animals, however, vitamin is transported to tissues and cells as a protein complex.

A study by Sidell et al. (1981) indicated that augmentation of mitogenic response of T lymphocytes may be dependent upon the availability of a restricted set of responder cells. In the presence of phytohemag-glutinin, RA increased the proliferation of cultured lymphocytes obtained from the thymus and tonsils of humans by 2.5 fold. In marked contrast, the presence of RA had no effect on the mitogenic response of lymphocytes obtained from either the spleen or peripherial blood. RA stimulated proliferation of immature lymphocytes, suggesting that RA promotes maturation of appropriate precursor cells. Such action would be consist-ent with findings that retinoids stimulate differentiation of epithelial (Fitton-Jackson and Fell, 1963) and embryonic cells (Strickland and Sawey, 1980) in culture.

The effect of vitamin A on allograft rejection has also been studied. Jurin and Tannock (1972) grafted skin from isologous males onto C57BL/6 female mice. When vitamin A was injected intraperitonealy 5 days before to 10 days after grafting, graft survival time was reduced from 35.5 days for controls to 24 days for animals treated with vitamin A. In addition, the time interval between the onset and completion of rejection was reduced to one half of the time for animals receiving vitamin A than for controls. The mechanism of action for vitamin A to increase rectivity to histoincompatible tissues is unclear. Malkovsky et al. (1983) reported that mice fed a conventional diet supplemented with vitamin A acetate

responded to semiallogenic cells in a host-versus-graft reaction, whereas

mice that had not received additional vitamin A supplementation did not.

It was then possible to transfer the increased reactivity to the alloanti-

gens by injecting lymph node cells derived from the vitamin A acetate-

treated mice into untreated syngeneic mice. The phenotype of the cells

responsible for the enhanced immune reactivity was identified as T helper

cells. Therefore, vitamin A's ability to increase reactivity of histocom-

patibility antigens may be mediated by its ability to increase the

proportion of helper cells in T cell populations.

## Immunoglobulin G

IgG is the most abundant immunoglobulin found in chicken serum. In

adult chickens, it has been found at a concentration of 5.29 mg/ml, with a

range of 3.7 to 8 mg/ml (Leslie and Martin, 1973). Chhabra and Goel

(1980) observed a similar average serum IgG concentration (5.09 mg/ml)

with a range of 1 to 13.5 mg/ml. Rees and Nordskog (1981) found higher

serum IgG concentrations within a range of 6.6 to 13.5 mg/ml. Because

this immunoglobulin appeared in the serum in greater concentrations than

any other immunoglobulin, it was assumed to be an equivalent of mammalian

IgG. However, antigenic analysis showed that human IgG does not cross-

react with chicken IgG (Leslie and Clem, 1969). In addition, the

molecular weight and carbohydrate content of the predominant immuno-

globulin in chicken serum are higher and the isoelectric point is lower

than mammalian IgG (Leslie and Clem, 1969). On the basis of these

physiochemical and immunochemical data, Leslie and Clem (1969) suggested

that the major immunoglobulin of chicken serum be designated as IgY since

it differed from mammalian IgG. However, this chicken immunoglobulin and human IgG share some biological homology in that they appear in serum in greater concentrations than other immunoglobulins, are the primary antibody of the secondary immune response, and have a longer half-life than IgM. Because of the important similarities between human and so-called chicken IgG, there seems to be ample justification for referring to the principle serum immunoglobulin of chickens as IgG.

Chicken IgG has a monomeric structure with a molecular weight between 165,000 (Orlans, 1968) and 206,000 daltons (Tenenhouse and Deutsch, 1966). It consists of two light chains and two heavy chains. The majority of chicken light chains (95%) are of one antigenic type. On the basis of amino acid sequence analysis, the light chains of the chicken immuno-globulin were shown to most closely resemble human lambda chain rather than kappa chain (Grant and Hood, 1971). Leslie (1977) reported a second isotype of chicken light chains. Whether the two light chains represent kappa and lambda isotypes or two lambda isotypes has not been determined. The existence of subclasses of chicken IgG have been reported using immunoelectrophoresis (Orlans and Rose, 1972).

Chicken IgG has a half-life of 1.5 to 4.1 days (Leslie and Clem, 1970). It is present in seminal plasma, alimentary tract secretions, feces (Leslie et al., 1971b) and oviduct washings (Orlans and Rose, 1972). The concentration of IgG in saliva of adult chickens ranged from .06 to .48 mg/ml as determined by Leslie and Martin (1974). More recently, Chhabra and Goel (1980) detected a concentration of .137 mg of IgG/ml in tracheal washings.

IgG is found in egg yolk (Rose et al., 1974). IgG is the vehicle of passive humoral immunity in neonatal chicks. High concentrations of IgG (5 mg/ml) have been detected in serum of 1-day-old chicks (Higgins and Calnek, 1975a). This is due to the transfer of IgG from the yolk sac to the serum of the chick. The high concentration of IgG in serum provides high titers of antibodies to a wide range of antigens to the newly hatched chick.

## Immunoglobulin M

The structure and properties of chicken IgM resemble those of mammalian IgM (Kobayashi and Hirai, 1980). Antigenic analysis of chicken IgM has supported the observation that it is similar to IgM of other species (Leslie and Clem, 1970). Two IgMs with different molecular weights have been detected in chicken serum (Kobayashi and Hirai, 1980). Seventy percent of the total serum IgM has a molecular weight of 860,000. The determined molecular weight is compatible with the basic structure of IgM as a pentamer of a molecular unit consisting of two heavy chains and two light chains. The minor serum IgM (approximately 30% of total IgM) has a molecular weight of 680,000 and is assumed to have a tetrameric structure. Reduced and alkylated chicken IgM has been shown to possess a J chain analogous to mammalian J chain (Kobayashi et al., 1973).

IgM is the first class of immunoglobulin to appear after exposure to most antigens. IgM antibodies to protein antigens have been detected 72 hours after inoculation. The IgM concentration reaches a peak at 4 to 8 days and then declines rapidly, being superseded by antibody of the IgG class (Khare et al., 1976).

The average serum concentration of IgM in adult chickens has been reported by a number of investigators to be within the range of .71 and 2.5 mg/ml (Leslie and Clem, 1970; Leslie and Martin, 1974; Rose et al., 1974; Lebacq-Verheyden et al., 1974; Higgins and Calnek, 1975b; Leslie et al., 1976; Chhabra and Goel, 1980). The serum IgG to IgM ratio was shown by Leslie and Clem (1970) to be 7.5 to 1. The half-life of IgM in adult chickens is 1.7 days with a range of 1.6 to 1.8 days (Leslie and Clem, 1970).

IgM has been detected in trace amounts in secretions. Chicken IgM has been found in seminal plasma (Leslie et al., 1971b; Watanabe et al., 1975), tears (Watanabe et al., 1975), oviduct washings and duodenal washings (Bienenstock et al., 1973b). Some investigators have detected IgM in bile (Bienenstock et al., 1973b; Leslie et al., 1976).

IgM has been detected in tracheal washings but the concentration was below the sensitivity of the radial immunodiffusion test used (Chhabra and Goel, 1980). In saliva of adult chickens, Lebacq-Verheyden et al. (1974) reported a concentration of .01 mg IgM per ml, whereas Leslie and Martin (1974) detected .085 mg/ml.

IgM is not found in yolk but is present in egg white. IgM is present in amniotic fluid of embyronating eggs and in the digestive tract of 19-day embryos (Rose et al., 1974). Maternal IgM found in embryo gut is derived from secretions acquired by the egg as it passes through the magnum area of the oviduct. IgM, however, is not usually present in the serum of newly hatched chicks (Leslie and Martin, 1973; Leslie et al.,

1976), although small amounts of IgM in serum were detected by Higgins and Calnek (1975b).

## Immunoglobulin A

Before 1972, all demonstrations of immunoglobulins in chicken secretions had shown the predominant component to be IgG with small amounts of IgM also present (Leslie et al., 1971b). Unlike humans and mammals, in which IgA is the predominant immunoglobulin in secretions, IgG was believed to constitute the basis of the local immune system in chickens. The first reports of a chicken immunoglobulin which exhibited antigenic differences from IgG and IgM were made in 1972 (Bienenstock et al., 1972; Lebacq-Verheyden et al., 1972; Orlans and Rose, 1972). This immunoglobulin was found in low concentrations in serum (Orlans and Rose, 1972; Leslie et al., 1976) but occurred preferentially in secretions such as bile (Bienenstock et al., 1972; Leslie et al., 1976; Rose et al., 1981), intestinal washings (Bienenstock et al., 1973b; Leslie and Martin, 1973; Watanabe and Kobayashi, 1974), cecal extracts (Orlans and Rose, 1972), oviduct washings (Orlans and Rose, 1972), and tracheal washings (Bienenstock et al., 1972; Orlans and Rose, 1972; Leslie and Martin, 1973; Katz et al., 1974). Because this immunoglobulin was the most prominent one in secretions, it was assumed to be an equivalent of mammalian IgA. However, studies which compared the antigenic relationship between so-called chick IgA and human or mammalian IgA (Porter and Parry, 1976; Parry and Porter, 1978; Hodge and Ambrosius, 1983) have failed to demonstrate immunological cross-reactivity of the proposed chicken IgA to the mammalian IgA class. The heavy chain of the chicken IgA is very different

from that of IgA of humans and mammals. Therefore, it has been suggested that this immunoglobulin of chicken secretions not be designated as IgA. While not all the properties of this chicken immunoglobulin are the same as those of the mammalian IgA, the two immunoglobulins have analagous functional properties. Thus, in this presentation, the term chicken IgA will be used.

Chicken monomeric IgA has the basic structure of IgM and IgG; two heavy chains combined with two light chains (Leslie and Martin, 1973; Kobayashi and Hirai, 1980). In serum, the IgA molecule is present in both large and small forms. The majority of serum IgA exists in the form of a dimer or greater (Leslie and Martin, 1973). The small component present in serum has a molecular weight of 180,000, which may correspond to a monomeric form (Leslie and Martin, 1973). The bulk of IgA in external secretions has a trimeric structure, with a molecular weight of 560,000 (Kobayashi and Hirai, 1980). A homologue of mammalian J chain has been shown by Kobayashi and Hirai (1980) to be present in polymeric IgA. No antigenic differences between polymeric IgA in secretions and serum have been detected (Bienenstock et al., 1972; Lebacq-Verheyden et al., 1972; Katz et al., 1974).

In the serum of adult chickens, reports of the average concentration of IgA vary from .31 to .61 mg/ml (Leslie and Martin, 1973; Rose et al., 1974; Chhabra and Goel, 1980) and the ratio of IgA to IgG is between .09 and .12 (Leslie and Martin, 1973; Rose et al., 1974; Chhabra and Goel, 1980). In tracheal washings, the concentration of IgA has been reported

as .12 mg/ml by Leslie and Martin (1973) and more recently by Chhabra and Goel (1980) as .027 mg/ml. An IgA to IgG ratio of 1.6 in tracheal washings was determined by Leslie and Martin (1973). However, Chhabra and Goel (1980) found a lower IgA to IgG ratio of .224. In intestinal washings, the concentration of IgA was shown by Leslie and Martin (1973) to be .11 mg/ml. IgA has also been detected in egg white (Rose et al., 1974). This is most probably maternal IgA acquired by the egg as it passes down the oviduct where the white is secreted. IgA has not been detected in serum of newly hatched chicks but has been found at approximately 12 days after hatch (Leslie et al., 1976).

Substantial amounts of IgA have been found in chicken bile (3-12 mg/ml) (Bienenstock et al., 1972; Lebacq-Verheyden et al., 1972; Leslie et al., 1976; Rose and Hesketh, 1979; Rose et al., 1981). Some IgG and IgM has also been found in bile (Bienenstock et al., 1973b; Watanabe et al., 1975). In bile, the IgA to IgG ratio is greater than 27 (Leslie and Martin, 1973).

Although bile is rich in IgA, its exact origin is not known. Evidence suggests that the immediate source of biliary IgA is polymeric IgA in the serum, which is actively and selectively transported from blood to bile by the liver. The concentration of IgA in chicken serum was shown to increase four-fold after bile ducts were ligated (Rose et al., 1981). This confirms that biliary IgA comes from blood in which the concentration is controlled by the active removal of polymeric IgA by the liver.

IgA in the circulation appears to be derived from IgA-secreting cells in the intestinal mucosa. Most of the plasma cells that synthesize IgA

are located immediately beneath the basement membrane of the epithelial cells, i.e., in the lamina propria of the gut. Two separate populations of mucosal lymphoid cells have been obtained from the small intestine of chickens (Arnaud-Battandier et al., 1980). One population was derived from within the epithelium and the second from within the lamina propria. The epithelium and lamina propria both contain immunoglobulin-secreting cells; however, the proportion of secretory cells is higher in the lamina propria. The lamina propria contains a high proportion of IgA-secreting cells but a high number of IgG-secreting cells are also present. Few IgM-secreting cells are detected in the mucosal lymphocytes.

The submucosal plasma cells secrete IgA into the local interstitial fluid. Some of the polymeric IgA is transported across the cytoplasm of the enterocytes and released into the lumen of the gut. The fine lymphatic vessels of chickens carry to the blood that portion of intestinal IgA that is not secreted directly into the lumen of the gut. The IgA in the circulation is carried across the hepatocyte cytoplasm and released into the biliary system.

The process of transporting polymeric IgA into secretions has been elucidated in rats and is mediated by secretory component (SC) (Hall, 1981). In the species studied to date, SC is a glycoprotein with a molecular weight of approximately 80,000, which is synthesized by epithelial cells and hepatocytes. During the process of secretion, polymeric IgA combines with SC. Monomeric IgA does not combine with SC. Once IgA is combined with SC, the complex is known as secretory IgA (SIgA). SC is a membrane receptor which transports polymeric IgA across

the cytoplasm in endocytic vesicles and discharges it intact into the biliary system or lumen of the gut.

Many researchers have attempted to establish the existence of chicken secretory component homologous to that which is associated with polymeric IgA in the secretions of mammals. These attempts, through immunochemical means, have yielded conflicting and inconsistent results (Katz et al., 1974; Rose et al., 1974; Watanabe and Kobayashi, 1974; Watanabe et al., 1975; Porter and Parry, 1976; Parry and Porter, 1978). The presence of chicken SC, however, has been demonstrated on the basis of its functional but not on its immunochemical homology with mammalian SC. Bienenstock et al. (1973b) were the first to demonstrate functional homology of chicken and mammalian SC. They found that chicken serum IgA would bind human SC labeled with $^{125}$I. A biliary preparation of chicken IgA, on the other hand, did not bind to human SC, suggesting that the SC binding sites present on bile IgA were already blocked by chicken SC (Bienenstock et al., 1973b). Additional studies have shown that radiolabeled human polymeric IgA injected intravenously into chickens is rapidly secreted into bile (Rose et al., 1981). If the mechanism of this process is due to the function of SC, as elucidated in rats, then SC would be synthesized by hepatocytes of the chicken. In a recent study (Peppard et al., 1983), intravenous injection of ($^{14}$C) fucose was given to chickens as a marker for newly synthesized glycoproteins. Unlabeled human IgA injected intravenously into chickens appeared in the bile combined wih a ($^{14}$C) fucose-labeled molecule. Chicken IgA in the same bile sample was similarly labeled. Purified chicken bile IgA was not transported in large

amounts across the chicken liver. These results provide further evidence that a molecule exists in chickens which behaves in a manner analagues to mammalian SC.

Evidence of a chicken IgA which is structurally and functionally close to mammalian IgA supports the existence of a secretory immune system in chickens. Local immunity is important since it acts as a barrier at mucosal surfaces where primary infections may occur, thereby interfering with further spread of the disease. The presence of immunoglobulins may provide the basis for local immunity. The predominant immunoglobulin in most chicken secretions is IgA, although significant amounts of IgG are also present. The role of IgA in protecting the mucosal surfaces of the chicken from infection has not been established. IgA has been demonstrated to be responsible for the majority of antiviral activity of the upper respiratory tract secretions of chickens infected with Newcastle disease virus (Parry and Aitken, 1973). However, the presence of IgA has been shown not to be essential for the development of local immunity in chickens (Ewert and Eidson, 1977). Neonatal bursectomy of one-day old chicks resulted in a selective depletion of IgA in serum and bile, whereas optimum IgG and IgM concentrations were maintained. After vaccination with NDV by either intramuscular or intratracheal routes, the bursectomized birds produced levels of serum hemagglutinating antibody and tracheal wash neutralizing antibody indistinguishable from those observed in shambursectomized chickens. All of the vaccinated, bursectomized chickens, when challenged with a virulent strain of NDV, remained healthy. It seems that depression of IgA synthesis does not prevent the development

of immunity in chickens and that other locally produced immunoglobulins
may protect the mucosa in the absence of IgA.

The interest in local immunity in chickens has been linked mainly to
an interest in achieving optimum protection from disease by proper
vaccination programs. A better immunity against respiratory infections
has been achieved by local immunization of the respiratory tract via
intranasal and intratracheal routes than by intramuscular or intravenous
vaccination (Ewert et al., 1979). The role of IgA and other classes of
immunoglobulins in providing enhanced disease protection as a result of
local vaccination has yet to be elucidated.

## Retinoic Acid

Retinoic acid (RA) is an oxidative product of the first step in the
metabolism of vitamin A. RA is not reduced to retinol or retinal. The
best indication that RA itself is an active, functional metabolite and not
an elimination metabolite is the discovery of two distinct intracellular
binding proteins specific for retinol or RA (Chytil and Ong, 1979).

The functions of vitamin A can only partially be fulfilled by RA. It
has been well-established that RA cannot replace retinal in its role in
vision (Dowling and Wald, 1960). Nor can RA satisfy the retinol require-
ments for maintaining the function of reproductive organs (Howell et al.,
1963; Moore, 1976). In addition, Chole and Quick (1978) found that the
development of tissues of the inner ear requires retinol and that RA will
not fulfill this metabolic requirement.

RA has been found to be active in vitamin A's role as a growth
promotant, since growth of animals can be started and stopped by giving RA

intermittently to or withholding RA from animals depleted of retinol (Lamb et al., 1974; Anzano et al., 1979). RA is also active in the differentiation and maintenance of epithelial and skeletal tissues. It is 10 times more active then retinol in suppressing keratinization of tracheas in organ culture (Sporn et al., 1975). RA was shown to be almost as active as retinol for stimulation of incorporation of mannose into glycoproteins (DeLuca et al., 1975).

Preliminary data indicate that RA is further metabolized to an as yet unidentified derivative. It is suggested that this metabolite can replace retinol in the phosphorylation and glysosylation pathways (Chen and Heller, 1977). It is not known at present whether both retinol and the RA derivative represent equally active forms of vitamin A in glycosylation reactions. It may be possible that the action of vitamin A in supporting growth and epithelial differentiation is mainly, if not solely, mediated through the unknown metabolite of RA.

The study of immune function in vitamin A deficient states is complicated by the fact that vitamin A deficiency is almost always associated with some degree of protein-energy malnutrition and other nutrient deficiencies, which are themselves known to exert negative effects on immune responses. In addition, vitamin A deficiency is often associated with concurrent infections which have profound effects on nutritional and immunological status. A rapid vitamin A deficiency, therefore, would be desirable in order to minimize general undernutrition and subclinical infection. This can be accomplished by supplementing and subsequently withdrawing RA from the diets of vitamin A deficient animals.

A state of deficiency can be expected to develop rapidly after RA

withdrawal from a vitamin A deficient diet since RA, unlike retinol or

retinal, cannot be stored in the body and is rapidly metabolized.

Krishnamurthy et al. (1963) reported that vitamin A deficient chicks

provided with daily doses of 100 ug of RA exhibited ataxia just before RA

was administered but exhibited no unsteadiness within a few hours.

Results of metabolic studies with $^{14}$C-RA, performed on these chicks,

showed that a significant quantity of the acid was present in the plasma,

liver and intestinal wall 3 hours after administration, but only traces

were detectable in the liver and intestine after 18 hours. Thus, daily

doses of 100 ug were so rapidly metabolized in the body that the duration

of effect was less than 24 hours. However, when RA was added directly to

the diet (6 mg/ml) so that a relatively continuous supply was obtained day

and night, chicks developed normally without any evidence of ataxia or eye

lesions.

Lamb et al. (1974) reported a successful method for inducing a rapid

vitamin A deficiency in rats. They fed weanling rats a vitamin A-free

diet until an early plateau in body weight was obtained. Then for the

next 18 days, a diet supplemented with 2 ug of RA/g of diet was fed. RA

was removed from the diet for the subsequent 10 days. The supplementation

and deprivation phases of RA feeding were repeated cyclically with the

same animals, which resulted in a rapid and complete vitamin A deficiency.

This is a convenient method for investigating direct effects of vitamin A

deficiencies because one can prevent the protein-energy malnutrition,

which often accompanies a vitamin A deficiency in animals, and thus
eliminate the need for pair-fed control animals.

## MATERIAL AND METHODS

### General

Five hundred male Indian River broiler chicks, obtained from a commercial hatchery, were used in one experiment conducted in the poultry research facilities at Iowa State University, Ames, Iowa.

The chicks were housed in six-deck brooder batteries at one day of age. Electric heaters were preset at a temperature of 35 C for the first week and reduced to 32, 29, 26, and 24 C when the chicks were one, two, three, and four weeks of age, respectively. Each battery had 9.5 mm raised, wire mesh floors. Feed and water were provided ad libitum from troughs adjacent to the pens. The water was changed daily. The dropping trays below the wire mesh floors were cleaned regularly to prevent the accumulation of excreta within the reach of the chicks. All pens received fluorescent lighting 24 hours a day during the first four weeks of the experiment.

At four weeks of age, chicks were transferred to slatted floor pens. The chicks remained in these pens until the experiment was terminated. The temperature of the pens was maintained within a range of 18 and 24 C. Feed and water were available ad libitum from adjacent troughs. Continuous fluorescent lighting was provided.

### Diets

At one day of age, broiler chicks were randomly allotted to 48 pens. Each pen contained 10 to 11 chicks at the start of the trial. All chicks in the 48 pens were fed a vitamin A-free experimental chick starter diet

(Table 1). The chick starter diet was formulated to meet the nutritive requirements of the broilers, based on values recommended by the National Research Council (NRC, 1977), except for vitamin A content.

After receiving the vitamin A-free diet for 13 days, pens of chicks were randomly assigned to three experimental diets. Chicks in each of eight pens were fed the chick starter diet supplemented with retinol (Hoffman-La Roche Inc., Ames, IA; 650,000 IU/gram) at a concentration of either 2 or 0.2 ug/g of diet (Table 1). The remaining 32 pens of chicks were fed the chick starter diet containing 2 ug all-trans retinoic acid (RA)/g of diet (Table 1). Retinoic acid (Sigma, St. Louis, MO) was dissolved in a minimal volume of ethanol and mixed with the vitamin premix before being added to the starter diet. The diets were prepared every three weeks to avoid prolonged storage of retinoic acid-containing feed.

At four weeks of age, chicks fed diets supplemented with 2 or 0.2 ug retinol/g diet were rerandomized into four pens for each treatment. Each pen contained 15 to 21 chicks. Chicks fed diets supplemented with 2 ug RA/g of diet were rerandomized into four pens of 14 to 16 chicks and 32 pens containing seven to nine chicks.

At 49 days of age, chicks fed 0.2 ug retinol/g of diet were changed to a vitamin A-free diet. Chicks fed diets containing 2 ug retinol/g of diet remained on this diet for the remainder of the experiment. Chicks fed diets supplemented with 2 ug RA/g of diet were divided into three dietary treatment groups. One group was changed to a vitamin A-free diet. A second group was fed a diet containing 0.2 ug RA/g of diet. Each of these diets was fed to 12 pens containing seven to nine chicks for the

Table 1. Composition of the test diets

| Ingredient | Vitamin A-free | Retinol diets 0.2 ug/g | Retinol diets 2.0 ug/g | Retinoic acid diets 0.2 ug/g | Retinoic acid diets 2.0 ug/g |
|---|---|---|---|---|---|
| Soybean meal (48% protein) (g) | 47.40 | 47.40 | 47.40 | 47.40 | 47.40 |
| Sucrose (g) | 43.08 | 43.08 | 43.08 | 43.08 | 43.08 |
| Soybean oil (g) | 5.00 | 5.00 | 5.00 | 5.00 | 5.00 |
| Dicalcium phosphate (g) | 2.80 | 2.80 | 2.80 | 2.80 | 2.80 |
| Ground limestone (g) | .65 | .65 | .65 | .65 | .65 |
| Salt premix[a] (g) | .30 | .30 | .30 | .30 | .30 |
| Vitamin premix[b] (mg) | 500.00 | 497.75 | 475.00 | 499.81 | 497.85 |
| Retinol (mg) | -- | 2.25[c] | 25.0[c] | -- | -- |
| Retinoic acid (mg) | -- | -- | -- | .19[d] | 2.15[d] |
| DL-methionine (g) | .27 | .27 | .27 | .27 | .27 |
| Total (g) | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 |

Calculated Analysis

| | | | | | |
|---|---|---|---|---|---|
| Metabolizable energy kcal/kg | 3183 | 3183 | 3183 | 3183 | 3183 |
| Protein, % | 23.13 | 23.13 | 23.13 | 23.13 | 23.13 |
| Calcium, % | .96 | .96 | .96 | .96 | .96 |

| | | | | | |
|---|---|---|---|---|---|
| Phosphorus, % | .81 | .81 | .81 | .81 | .81 |
| TSA,[e] % | .96 | .96 | .96 | .96 | .96 |
| Vitamin A, IU/kg | 0 | 675 | 7500 | 0 | 0 |
| Retinoic acid, IU/kg | 0 | 0 | 0 | 675 | 7500 |

[a]Supplied per kg of diet:   21.6 mg manganese, 50 mg zinc, 100 mg iron, 24 mg copper, 2.55 gm sodium chloride, and  .1 ppm selenium.

[b]Supplied per kg of diet:   1500 IU vitamin $D_3$, 6 IU vitamin E, 20 ug vitamin $B_{12}$, 1 mg vitamin K, 6 mg riboflavin, 22 mg pantothenic acid, 75 mg niacin, 400 mg choline chloride.

[c]30,000 IU retinol/g.

[d]100% Trans vitamin A acid.

[e]Total sulfur amino acids.

remainder of the experiment. The third group, consisting of 14 to 16

chicks in each of four pens and seven to nine chicks in each of eight

pens, remained on the diet supplemented with 2 ug RA/g of diet and served

as a positive control for the two RA-deficient groups.

Average body weights and feed consumption were determined weekly from

2 to 7 weeks of age. Individual body weights were also recorded before

chicks were killed for tissue sampling.

### Newcastle Disease Virus Vaccination

At one day of age, 29 chicks were killed to obtain blood. Serum was

analyzed for passively acquired maternal antibody to Newcastle disease

virus (NDV) by hemagglutination inhibition (HI) test (see Appendix A for

procedure).

At 14 days of age, all chicks in the three dietary treatment groups

were vaccinated with 0.1 ml of a live B-1 strain ($10^{9.3}ELD_{50}$) (embryo-

lethal dose 50%) of NDV (obtained through the courtesy of Dr. Melvin S.

Hofstad, Veterinary Medical Research Institute, Iowa State University,

Ames, IA). The virus suspension was administered intranasally and

intraocularly dropwise with a syringe equipped with a 1.27 cm no. 26

needle.

At 14, 21, and 28 days of age, two chicks from eight pens receiving

each of the three dietary treatments were sampled for blood. At 35 and 42

days of age, four chicks from four pens receiving each of the three

dietary treatments were sampled for blood. Blood samples obtained weekly

from 14 to 42 days of age were obtained by puncture of the wing vein.

At 49 days of age, seven or eight chicks from each of four pens from the three dietary treatment groups were bled and killed for tissue sampling. The chicks sampled were those from pens containing more than 14 chicks. These included all pens receiving 2 and 0.2 ug retinol/g of diet and only those pens containing 14 to 16 chicks that were receiving diets supplemented with 2 ug RA/g of diet.

The remaining chicks in each of these pens were revaccinated with NDV four days after final diet changes were made (53 days of age). Similarly, chicks in pens containing seven to nine chicks that had been receiving diets supplemented with 2 ug RA/g of diet were revaccinated at two, four, or six days after final diet assignments were made (51, 53, or 55 days of age, respectively).

Chicks were revaccinated with a formal-inactivated La Sota strain ($10^{9.3}$ELD$_{50}$) of NDV. The vaccine was administered intranasally and intraocularly as described previously. At these times, chicks were also vaccinated intramuscularly with 1 ml of virus suspension mixed with an equal volume of Freunds complete adjuvant (Gibco, Grand Island, NY). The killed vaccine was injected into the pectoral muscle in equal doses on both sides of the sternal septum.

Eight days after chicks received their second vaccination of NDV, they were bled and killed for tissue sampling (59, 61, or 63 days of age).

## Tissue Collection

Food was withdrawn from chicks for 48 hours before blood, bile, and tissues were collected. Five ml of blood were obtained from each chick by heart probe by using a 3.81 cm, no. 20 short bevel needle. All blood

samples collected during the experiment were allowed to clot at room temperature. The serum was removed with a pipette and stored at -20 C until assayed. Chicks were killed by injecting .5 ml T-61 Euthanasia solution (American Hoechst Corp., Somerville, NJ) into the heart. Immediately after death, 20 cm of trachea from the larynx to the syrinx were removed. Similarly, 20 cm of intestine, 10 cm above and 10 cm below the yolk stalk, were removed. The tissues, including their contents, were homogenized in 10 ml of cold (4 C) phosphate-buffered saline (PBS, pH 7.2) (containing .01% $NaN_3$ and $10^{-3}$ m phenylmethanesulfonyl fluoride). The homogenate was centrifuged and the supernatant from each individual sample was frozen at -20 C until analyzed (see Appendix B for procedure).

Livers were removed, weighed, and frozen until analyzed for retinol by a modified Carr-Price assay procedure (see Appendix J for procedure).

One to two milliliter of bile was aspirated directly from the gallbladder of each chick using a 2.54 cm, no. 21 short bevel needle and stored at -20 C until analyzed.

## Total Protein

Total protein concentrations of samples of bile, serum, and tissue supernatants were determined by the method of Lowry et al. (1951). Bovine serum albumin was used as the protein standard.

## Immunoglobulins

Chicken IgG and IgM concentrations in individual serum and tissue samples were estimated by radial immunodiffusion by using goat antiserum

monospecific for chicken IgG (Pel-Frey, Rogers, AR) an IgM (Miles Laboratories, Elkhart, IN), respectively (see Appendix C for procedure).

Chicken IgA concentrations in serum, bile, and tissues were determined by rocket immunoelectrophoresis using goat antiseria monospecific for chicken IgA (Miles Laboratories, Elkhart, IN) (see Appendix D for procedure).

Immunoglobulin (Ig) concentrations in serum and bile were expressed as mg Ig/ml. To aid in comparison of values among individuals having different tissue weights and protein contents, the amount of immunoglobulin in each ml of tissue supernatant was expressed as a function of tissue weight (mg Ig/g of tissue).

## Antibody Titer to Newcastle Disease Virus Vaccinations

All individual serum samples obtained during the experiment.were analyzed for antibody titer to Newcastle disease virus by hemaglutination inhibition test (see Appendix A for procedure). Geometric mean antibody titers (GMT) were calculated for each treatment group.

## Statistical Analysis

All data collected were analyzed statistically by analysis of variance. The data collected in all the experiments were processed by the Statistical Analysis System (SAS) (Barr et al., 1979).

Independent orthogonal comparisons were made to test differences between treatment means within a particular age.

RESULTS

On the 13th day after consumption of vitamin A-free diet, chicks exhibited incoordination. At this first sign of vitamin A deficiency, all of the chicks were fed diets supplemented with vitamin A in the form of retinol or retinoic acid. Body weights of chicks after the start of supplementation of diets with vitamin A to 7 weeks of age are presented in Table 2 (Appendix K1-16). Despite the randomization of pen assignments to dietary treatments, initial body weights of chicks assigned to diets containing 2 ug RA/g of diet were significantly (p<.007) greater compared with chicks assigned to diets containing 2 ug retinol/g of diet. Body weights of chicks fed diets supplemented with 2 ug RA/g of diet continued to be significantly greater at 3 and 4 weeks of age as compared with retinol controls. Differences between body weights of RA control and retinol control chicks were not significantly different at 5, 6, and 7 weeks of age. Chicks consuming diets containing 0.2 ug retinol/g of diet had body weights similar to those of chicks fed 2 ug retinol/g of diet until 5 weeks of age. At 5 weeks of age, it became apparent that vitamin A deficiency was impairing growth of chicks. Chicks fed 0.2 ug retinol/g of diet had significantly lower body weights at 5, 6, and 7 weeks of age as compared with chicks fed diets supplemented with 2 ug retinol/g of diet.

There were no significant differences in feed consumption among the three treatment groups from 2 to 5 weeks of age (Table 3, Appendix K1-4). At 6 weeks of age, there was a small but significant decrease (p<.02) in feed consumed by chicks fed 0.2 ug retinol/g of diet as compared with

Table 2. Average body weights of chicks as influenced by dietary retinol and retinoic acid supplementation from 2 to 7 weeks of age

| Vitamin A source | ug/g diet | Body weight (g) Weeks of age | | | | | |
|---|---|---|---|---|---|---|---|
| | | 2 | 3 | 4 | 5 | 6 | 7 |
| Retinol | 2.0[a] | 184 | 366 | 651 | 1002 | 1462 | 1913 |
| | 0.2[a] | 184 | 362 | 634 | 943 | 1319 | 1541 |
| Retinoic acid | 2.0[b] | 193 | 406 | 694 | 1010 | 1410 | 1793 |
| C.V.[c] | | 4.2 | 4.3 | 3.6 | 3.9 | 4.2 | 9.6 |
| Level of significance | | | | | | | |
| Treatment | | .002 | .001 | .001 | .009 | .005 | .01 |
| Retinol 2 vs. retinoic acid 2 | | .007 | .001 | .001 | NS[d] | NS | NS |
| Retinol 2 vs 0.2 | | NS | NS | NS | .04 | .002 | .004 |

[a]There were 8 replicates from 2 to 4 weeks of age and 4 replicates from 5 to 7 weeks of age.

[b]There were 32 replicates from 2 to 4 weeks of age and 36 replicates from 5 to 7 weeks of age.

[c]Coefficient of variation.

[d]No significant differences among treatment means.

Table 3. Average daily feed consumption of chicks as influenced by dietary retinol and retinoic acid supplementation from 2 to 7 weeks of age

| Vitamin A source | ug/g diet | Feed consumed (g/chick/day) Weeks of age | | | | | |
|---|---|---|---|---|---|---|---|
| | | 2 | 3 | 4 | 5 | 6 | 7 |
| Retinol | $2.0^a$ | 26 | 43 | 68 | 96 | 135 | 141 |
| | $0.2^a$ | 27 | 42 | 68 | 95 | 120 | 130 |
| Retinoic acid | $2.0^b$ | 27 | 44 | 68 | 95 | 128 | 129 |
| C.V.$^c$ | | 6.9 | 5.6 | 3.9 | 9.1 | 6.8 | 10.8 |
| Level of significance | | | | | | | |
| Treatment | | NS$^d$ | NS | NS | NS | .07 | NS |
| Retinol 2 vs. retinoic acid 2 | | NS | NS | NS | NS | NS | NS |
| Retinol 2 vs 0.2 | | NS | NS | NS | NS | .02 | NS |

[a]There were 8 replicates from 2 to 4 weeks of age and 4 replicates from 5 to 7 weeks of age.

[b]There were 32 replicates from 2 to 4 weeks of age and 36 replicates from 5 to 7 weeks of age.

[c]Coefficient of variation.

[d]No significant differences among treatment means.

chicks fed 2 ug retinol/g of diet (Appendix K5). The decrease in average feed consumption by retinol deficient chicks was observed also at 7 weeks of age, but differences among treatments were not significant (Appendix K6). Feed consumption by chicks fed diets supplemented with 2 ug retinol or RA/g of diet was similar throughout the treatment period.

Feed consumption per unit of gain determined for each of the three treatment groups from 2 to 7 weeks of age is shown in Table 4. Statistical analysis of the amount of feed that the chicks consumed, during weeks 3 and 4, to gain 1 g of body weight indicated significant ($p < .05$) treatment effects (Appendix K2-3). Independent orthogonal comparisons were made between the retinol control and RA control groups and between the retinol control and retinol deficient groups. However, neither of these comparisons yielded significant differences among treatment groups at any time during the 2 to 7 week period (Appendix K1-6).

Serum samples obtained from chicks from 2 to 7 weeks of age were analyzed for protein. The results of these analyses are shown in Table 5. Statistical analysis confirmed that vitamin A source and concentration had no significant effect on mg of protein/ml of serum (Appendix K7-12).

After vaccinating 2-week-old chicks intranasally and intraocularly with a live B1 strain of NDV, the concentration of immunoglobulins, including IgG, IgM, and IgA, in serum was determined. As shown in Table 6, serum IgG concentrations expressed as mg/ml of serum increased continuously from 2 to 7 weeks of age. The increase occurred in all chicks regardless of dietary treatment. Three weeks after exposure to NDV (5

Table 4. Average feed efficiency of chicks as influenced by dietary retinol and retinoic acid supplementation from 2 to 7 weeks of age

| Vitamin A source | ug/g diet | Feed/gain | | | | | |
|---|---|---|---|---|---|---|---|
| | | Weeks of age | | | | | |
| | | 2 | 3 | 4 | 5 | 6 | 7 |
| Retinol | $2.0^a$ | 1.71 | 1.61 | 1.77 | 1.93 | 2.05 | 2.19 |
| | $0.2^a$ | 1.69 | 1.70 | 1.80 | 2.2 | 2.28 | 2.46 |
| Retinoic acid | $2.0^b$ | 1.63 | 1.49 | 1.66 | 2.14 | 2.26 | 2.27 |
| C.V.$^c$ | | 8.7 | 9.7 | 8.2 | 10.3 | 12.2 | 24.9 |
| Level of significance | | | | | | | |
| Treatment | | NS$^d$ | .002 | .017 | NS | NS | NS |
| Retinol 2 vs. retinoic acid 2 | | NS | NS | NS | NS | NS | NS |
| Retinol 2 vs 0.2 | | NS | NS | NS | NS | NS | NS |

[a]There were 8 replicates from 2 to 4 weeks of age and 4 replicates from 5 to 7 weeks of age.

[b]There were 32 replicates from 2 to 4 weeks of age and 36 replicates from 5 to 7 weeks of age.

[c]Coefficient of variation.

[d]No significant differences among treatment means.

Table 5. Total serum protein of chicks as influenced by dietary retinol and retinoic acid supplementation from 2 to 7 weeks of age

| Vitamin A source | ug/g diet | Total serum protein (mg/ml) Weeks of age | | | | | |
|---|---|---|---|---|---|---|---|
| | | $2^a$ | 3 | 4 | $5^b$ | 6 | 7 |
| Retinol | 2.0 | 42 | 45 | 33 | 46 | 55 | 36 |
| | 0.2 | 41 | 43 | 33 | 43 | 52 | 38 |
| Retinoic acid | 2.0 | 39 | 45 | 32 | 49 | 62 | 40 |
| C.V.$^c$ | | 7.6 | 5.1 | 9.3 | 12.8 | 19.3 | 7.2 |
| Level of significance | | | | | | | |
| Treatment | | NS$^d$ | NS | NS | NS | NS | NS |
| Retinol 2 vs. retinoic acid 2 | | NS | NS | NS | NS | NS | NS |
| Retinol 2 vs 0.2 | | NS | NS | NS | NS | NS | NS |

[a]There were 8 replicates per treatment from 2 to 4 weeks of age.

[b]There were 4 replicates per treatment from 5 to 7 weeks of age.

[c]Coefficient of variation.

[d]No significant differences among treatment means.

Table 6. Serum IgG concentrations of chicks as influenced by dietary retinol and retinoic acid supplementation from 2 to 7 weeks of age

| Vitamin A source | ug/g diet | Serum IgG (mg/ml) | | | | | |
|---|---|---|---|---|---|---|---|
| | | Weeks of age | | | | | |
| | | 2[a] | 3 | 4 | 5[b] | 6 | 7 |
| Retinol | 2.0 | 1.10 | 1.45 | 3.30 | 3.48 | 5.71 | 6.62 |
| | 0.2 | 1.12 | 1.45 | 3.02 | 4.12 | 7.64 | 7.51 |
| Retinoic acid | 2.0 | 1.11 | 1.52 | 2.87 | 4.70 | 7.16 | 7.88 |
| C.V.[c] | | 39 | 31 | 26 | 15 | 25 | 15 |
| Level of significance | | | | | | | |
| Treatment | | NS[d] | NS | NS | .062 | NS | .029 |
| Retinol 2 vs. retinoic acid 2 | | NS | NS | NS | .021 | NS | .011 |
| Retinol 2 vs 0.2 | | NS | NS | NS | NS | NS | .05 |

[a]There were 8 replicates per treatment from 2 to 4 weeks of age.

[b]There were 4 replicates per treatment from 2 to 4 weeks of age.

[c]Coefficient of variation.

[d]No significant differences among treatment means.

weeks of age) average serum IgG concentrations were greater in retinol deficient chicks and chicks fed diets containing adequate retinoic acid than in chicks fed diets containing adequate retinol. The relative differences among the three treatment groups persisted through 7 weeks of age at which time serum IgG of chicks fed 2 ug retinol/g of diet was significantly less than that of chicks in the other two treatment groups (Appendix K7-12).

Average serum IgM concentrations in chicks from all treatment groups increased linearly to 6 weeks of age (4 weeks after primary vaccination with NDV) (Table 7). At 7 weeks of age, a decline in IgM concentration from the previous week occurred in all treatment groups. Nevertheless, significant diet effects were observed (Appendix K7-12). Average IgM concentrations were greater in serum of chicks fed retinol deficient or RA adequate diets than in the serum of retinol controls. The pattern of treatment effects on serum IgM was similar to that observed with IgG but occurred one week earlier (at 4 instead of 5 weeks of age). At 4 weeks of age, serum IgM concentrations were significantly greater in chicks fed RA adequate and retinol deficient diets than in chicks fed retinol control diets. At 5 weeks of age, single degree of freedom comparisons showed that the increase in serum IgM concentrations above retinol controls was significant ($p < .028$) for RA fed chicks but not for retinol deficient chicks. The relative differences among treatment groups were observed again at 6 weeks of age, but differences among treatments were not statistically significant. Statistical analysis of average serum IgM concentrations at 7 weeks of age indicated highly significant ($p < .003$)

Table 7. Serum IgM concentrations of chicks as influenced by dietary retinol and retinoic acid supplementation from 2 to 7 weeks of age

| Vitamin A source | ug/g diet | Serum IgM (mg/ml) Weeks of age | | | | | |
|---|---|---|---|---|---|---|---|
| | | $2^a$ | 3 | 4 | $5^b$ | 6 | 7 |
| Retinol | 2.0 | .516 | 1.72 | 1.66 | 2.45 | 2.78 | 1.69 |
| | 0.2 | .764 | 2.08 | 2.07 | 2.84 | 3.54 | 2.28 |
| Retinoic acid | 2.0 | .572 | 1.88 | 2.07 | 3.82 | 3.02 | 2.33 |
| C.V.$^c$ | | 50 | 25 | 23 | 24 | 24 | 10 |
| Level of significance | | | | | | | |
| Treatment | | NS$^d$ | NS | .109 | .068 | NS | .004 |
| Retinol 2 vs. retinoic acid 2 | | NS | NS | .063 | .028 | NS | .003 |
| Retinol 2 vs 0.2 | | NS | NS | .074 | NS | NS | .004 |

[a]There were 8 replicates per treatment from 2 to 4 weeks of age.

[b]There were 4 replicates per treatment from 5 to 7 weeks of age.

[c]Coefficient of variation.

[d]No significant differences among treatment means.

differences among treatment groups with chicks fed 2.0 ug retinol/g of diet having the lowest concentration.

Average serum IgA concentrations of chicks from the three treatment groups resembled IgM in response to exposure to NDV (Table 8, Appendix K7-12). Serum IgA increased linearly from 2 to 6 weeks of age and then declined at 7 weeks of age. The pattern of response of serum IgA concentrations to the three dietary treatments also was similar to that observed for serum IgG and IgM. Average IgA concentrations were greater in retinol deficient chicks and RA fed chicks at 4, 5, 6, and 7 weeks of age as compared with retinol controls. Single degree of freedom comparisons indicated significant differences between retinol adequate and retinol deficient chicks at 4, 5, and 7 weeks of age. Significant ($p < .006$) differences between the two sources of dietary vitamin A occurred only at 7 weeks of age.

Passively acquired maternal antibodies to NDV were detected in the serum of day-old chicks used in the study. At 1 day of age, the serum geometric mean titer (GMT) was $32 \pm 34$ in 29 chicks tested. The concentration of maternal antibody declined to an average GMT of 5.5 at 2 weeks of age (Table 9), at which time chicks were given an initial vaccination of NDV. Fourteen days (4 weeks of age) after the primary exposure to NDV, the serum GMT, averaged over all treatment groups, reached a maximum of 9 and declined to 6.5 at 6 weeks of age. The GMT to NDV in serum of chicks sampled at 7 weeks of age was 25. There were no significant effects of diet treatments in serum antibody titer to NDV from 2 to 7 weeks of age (Appendix K7-12).

Table 8. Serum IgA concentrations of chicks as influenced by dietary retinol and retinoic acid supplementation from 2 to 7 weeks of age

| Vitamin A source | ug/g diet | Serum IgA (mg/ml) | | | | | |
| | | Weeks of age | | | | | |
| | | 2[a] | 3 | 4 | 5[b] | 6 | 7 |
| Retinol | 2.0 | .393 | .406 | .668 | .90 | 1.03 | .388 |
| | 0.2 | .417 | .492 | .953 | 1.31 | 1.18 | .722 |
| Retinoic acid | 2.0 | .355 | .428 | .870 | 1.02 | 1.19 | .855 |
| C.V.[c] | | 42 | 28 | 26 | 23 | 25 | 28 |
| Level of significance | | | | | | | |
| Treatment | | NS[d] | NS | .079 | .100 | NS | .016 |
| Retinol 2 vs. retinoic acid 2 | | NS | NS | NS | NS | NS | .006 |
| Retinol 2 vs 0.2 | | NS | NS | .03 | .05 | NS | .032 |

[a]There were 8 replicates per treatment from 2 to 4 weeks of age.

[b]There were 4 replicates per treatment from 5 to 7 weeks of age.

[c]Coefficient of variation.

[d]No significant differences among treatment means.

Table 9. Serum hemagglutination inhibition titer as influenced by dietary retinol and retinoic acid supplementation from 2 to 7 weeks of age after vaccination with Newcastle disease virus B-1

| Vitamin A source | ug/g diet | Geometric mean titer[a] | | | | | |
| | | Weeks of age | | | | | |
| | | 2[b] | 3 | 4 | 5[c] | 6 | 7 |
|---|---|---|---|---|---|---|---|
| Retinol | 2.0 | 5.88 | 8.63 | 9.75 | 7.50 | 6.50 | 27.25 |
| | 0.2 | 5.63 | 9.00 | 10.13 | 6.50 | 6.25 | 22.00 |
| Retinoic acid | 2.0 | 5.25 | 8.88 | 7.00 | 7.50 | 6.50 | 21.50 |
| C.V.[d] | | 27.15 | 40.50 | 57.46 | 24.17 | 18.55 | 34.38 |
| Level of significance | | | | | | | |
| Treatment | | NS[e] | NS | NS | NS | NS | NS |
| Retinol 2 vs. retinoic acid 2 | | NS | NS | NS | NS | NS | NS |
| Retinol 2 vs 0.2 | | NS | NS | NS | NS | NS | NS |

[a]Geometric mean titers (GMT) represent the reciprocal of the greatest dilution of serum showing inhibition of agglutination with antigen.

[b]There were 8 replicates per treatment from 2 to 4 weeks of age.

[c]There were 4 replicates per treatment from 5 to 7 weeks of age.

[d]Coefficient of variation.

[e]No significant differences among treatment means.

Seven week body weight data previously shown in Table 2 are presented again in Table 10 and are listed in the column headed by Day 0 of vitamin A withdrawal. Also included in Table 10 are average body weights of chicks 8 days after they were given a second vaccination of NDV. Second vaccinations of NDV were given 2, 4, or 6 days after final diet changes were made (Days 2, 4, and 6, respectively). Average body weights obtained at 7 weeks of age (Day 0) were related, therefore, to the effect of initial dietary treatments before final diet assignments and second vaccinations of NDV were given. As mentioned previously, chicks fed diets supplemented with 0.2 ug retinol/g of diet, from 2 to 7 weeks of age, had significantly ($p < .004$) lower average body weights at 7 weeks of age (Day 0) than chicks fed diets supplemented with 2 ug retinol/g (Appendix K6). Four days after retinol deficient chicks were changed to a vitamin A-free diet, they and chicks continuously fed 2 ug of retinol or RA/g of diet were vaccinated with NDV. Body weights of the retinol deficient chicks 8 days after vaccination remained significantly ($p < .003$) lower than body weights of retinol control chicks (Appendix K13). Chicks that remained on RA adequate diets (2 ug/g of diet) maintained similar body weights as chicks fed the retinol adequate diet. When chicks previously fed 2 ug RA/g of diet were changed to a diet supplemented with 0.2 ug RA/g of diet, body weights remained nearly the same as that of chicks continued on diets supplemented with 2 ug RA/g of diet. However, when all RA was removed from diets of chicks previously fed adequate RA, a rapid decline in weight gain occurred. The longer the duration between removal of RA from diets and time of vaccination, the more severe the growth depression.

Table 10. Average body weights of chicks as influenced by dietary retinol and retinoic acid supplementation before and 8 days after second vaccination with Newcastle disease virus—La Sota[a]

| Vitamin A source | ug/g diet | N[b] | Body weight (g) Days after vitamin A withdrawal | | | |
|---|---|---|---|---|---|---|
| | | | 0 | 2 | 4 | 6 |
| Retinol | 2.0 | 4 | 1910 | ND[c] | 2260 | ND |
| | 0.2 | 4 | 1540 | ND | 2010 | ND |
| Retinoic acid | 2.0 | 4 | 1790 | 2050 | 2210 | 2170 |
| | 0.2 | 4 | | 2000 | 2090 | 2080 |
| | 0 | 4 | | 1790 | 1700 | 1650 |
| C.V.[d] | | | 5 | 6 | 5 | 7 |
| Level of significance | | | | | | |
| Treatments | | | .01 | .023 | .0001 | .001 |
| Retinol 2 vs. retinoic acid 2 | | | NS[e] | ND | NS | ND |
| Retinol 2 vs. 0.2 | | | .004 | ND | .003 | ND |
| Retinoic acid 2 vs. 0.2 | | | | NS | NS | NS |
| Retinoic acid 2 vs. 0 | | | | .01 | .0001 | .0005 |

[a]Vaccinations were given 2, 4, or 6 days after vitamin A was withdrawn from diets.

[b]N=Number of replicates.

[c]Not determined.

[d]Coefficient of variation.

[e]No significant difference among treatment means.

Mean vitamin A concentrations, in ug/g of tissue, were determined in livers of chicks before (Day 0) and 8 days after vaccination (Day 4) with NDV. These data are presented in Table 11. Chicks fed a vitamin A-free diet until 2 weeks of age and then fed a diet supplemented with RA, had no detectable liver vitamin A when determined at 7 weeks of age (Day 0) or after vaccination (Day 4). Chicks fed diets supplemented with 2 ug retinol/g of diet had an average of 18.5 and 21.6 ug vitamin A/g of liver before and after vaccination, respectively. The liver vitamin A concentrations from retinol controls were considerably greater than of chicks fed 0.2 ug retinol/g of diet. Chicks of the latter treatment group had only 2.89 ug of vitamin A/g of liver at 7 weeks of age (Day 0). Liver vitamin A of chicks from this group declined to 1.27 ug/g of liver 12 days after the feeding of a vitamin A-free diet began.

Total serum protein concentration were also determined in chicks after they received a second vaccination of NDV. These data as well as total serum protein data at 7 weeks of age are presented in Table 12. Irrespective of dietary treatment, total serum protein was greater in chicks 8 days after vaccination with NDV (Days 2, 4, and 6) than before vaccination (Day 0). Statistical analysis of the data indicated a significant ($p < .0002$) increase in serum protein in chicks fed no retinoic acid (62 mg/ml) as compared with the retinoic acid controls (50 mg/ml). This diet effect was observed only in chicks vaccinated at Day 4. There were no significant differences among any of the treatment groups observed at other times (Appendix K14-16).

Table 11.   Liver vitamin A concentrations of chicks as influenced by
            dietary retinol and retinoic acid supplementation before and
            8 days after second vaccination with Newcastle disease
            virus-La Sota[a,b]

| Vitamin A source | ug/g diet | N[c] | Vitamin A ug/g liver | |
| | | | Days after vitamin A withdrawal | |
| | | | 0 | 4 |
|---|---|---|---|---|
| Retinol | 2.0 | 4 | 18.5±2.5 | 21.6±2.9 |
| | 0.2 | 4 | 2.89±0.7 | 1.27±1.1 |
| Retinoic acid | 2.0 | 4 | 0 | 0 |
| | 0.2 | 4 | | 0 |
| | 0 | 4 | | 0 |

[a]Chicks were vaccinated 4 days after vitamin A was withdrawn from diets.

[b]Means ± standard deviations.

[c]N=Number of replicates.

A second administration of antigen after a primary vaccination can
result in a greater rate of antibody synthesis.  To test this response,
IgG, IgM, and IgA concentrations were determined 8 days after chicks on
various dietary treatments were revaccinated with NDV.  As indicated in
Table 13, serum IgG concentrations in chicks from all treatment groups
increased after a second vaccination of NDV (Days 2, 4, and 6).  The serum
IgG concentrations were well above those attained by chicks before
vaccination (Day 0).  The average IgG concentration in serum of chicks fed
diets supplemented with 2 ug RA/g of diet (13.2 mg/ml) was significantly
($p < .02$) greater, after vaccination, than in chicks fed diets supplemented

Table 12. Total serum protein of chicks as influenced by dietary retinol and retinoic acid supplementation before and 8 days after second vaccination with Newcastle disease virus–La Sota[a]

| Vitamin A source | ug/g diet | N[b] | Total serum protein (mg/ml) | | | |
| | | | Days after vitamin A withdrawal | | | |
| | | | 0 | 2 | 4 | 6 |
|---|---|---|---|---|---|---|
| Retinol | 2.0 | 4 | 36 | ND[c] | 50 | ND |
| | 0.2 | 4 | 38 | ND | 52 | ND |
| Retinoic acid | 2.0 | 4 | 40 | 56 | 50 | 51 |
| | 0.2 | 4 | | 51 | 52 | 52 |
| | 0 | 4 | | 56 | 62 | 56 |
| C.V.[d] | | | 7 | 10 | 6 | 12 |

Level of significance

| | | | | | | |
|---|---|---|---|---|---|---|
| Treatments | | | NS[e] | NS | .0005 | NS |
| Retinol 2 vs. retinoic acid 2 | | | NS | ND | NS | ND |
| Retinol 2 vs. 0.2 | | | NS | ND | NS | ND |
| Retinoic acid 2 vs. 0.2 | | | | NS | NS | NS |
| Retinoic acid 2 vs. 0 | | | | NS | .0002 | NS |

[a]Vaccinations were given 2, 4, or 6 days after vitamin A was withdrawn from diets.

[b]N=Number of replicates.

[c]Not determined.

[d]Coefficient of variation.

[e]No significant difference among treatment means.

Table 13. Serum IgG concentrations of chicks as influenced by dietary retinol and retinoic acid supplementation before and 8 days after second vaccination with Newcastle disease virus–La Sota[a]

| Vitamin A source | ug/g diet | N[b] | Serum IgG (mg/ml) Days after vitamin A withdrawal | | | |
|---|---|---|---|---|---|---|
| | | | 0 | 2 | 4 | 6 |
| Retinol | 2.0 | 4 | 6.6 | ND[c] | 9.9 | ND |
| | 0.2 | 4 | 7.5 | ND | 14.8 | ND |
| Retinoic acid | 2.0 | 4 | 7.9 | 12.9 | 13.2 | 11.4 |
| | 0.2 | 4 | | 15.9 | 13.9 | 15.5 |
| | 0 | 4 | | 18.8 | 16.8 | 16.8 |
| C.V.[d] | | | 8 | 29 | 13 | 15 |

Level of significance

| Treatments | .029 | NS[e] | .001 | .02 |
|---|---|---|---|---|
| Retinol 2 vs. retinoic acid 2 | .011 | ND | .020 | ND |
| Retinol 2 vs. 0.2 | .051 | ND | .002 | ND |
| Retinoic acid 2 vs. 0.2 | | NS | NS | .028 |
| Retinoic acid 2 vs. 0 | | .095 | .01 | .008 |

[a]Vaccinations were given 2, 4, or 6 days after vitamin A was withdrawn from diets.

[b]N=Number of replicates.

[c]Not determined.

[d]Coefficient of variation.

[e]No significant difference among treatment means.

with an equivalent amount of retinol (9.9 mg/ml) (Appendix K15). Removing vitamin A from the diets of retinoic acid controls and retinol deficient chicks resulted in greater increases in serum IgG concentrations as compared with average IgG concentrations in serum of the vitamin A controls. The increase in IgG concentration was greater in chicks fed a totally vitamin A deficient diet than in chicks fed a partially deficient diet (0 vs. 0.2 ug RA/g of diet, respectively). The same pattern of response among dietary treatment groups was observed whether chicks were vaccinated at 2, 4, or 6 days after vitamin A was removed from the diet. Significant ($p<.028$) differences in serum IgG concentrations between chicks fed diets supplemented with 0.2 and 2 ug RA/g of diet occurred only when the second vaccination was administered 6 days after vitamin A was withdrawn from the diet (Appendix K16). Significant differences in serum IgG concentrations between chicks fed diets supplemented with 0 and 2 ug RA/g of diet occurred regardless of time of vaccination (Appendix K14-16).

The pattern of response to different dietary treatments for serum IgM concentrations after the second vaccination was similar to that observed for serum IgG (Table 14). Serum IgM concentrations were greater in all chicks after revaccination than before vaccination. Chicks fed adequate dietary vitamin A in the form of RA had greater average serum IgM concentrations (3.87 mg/ml), after vaccination, than chicks fed an equal concentration of vitamin A in the form of retinol (2.94 mg/ml). This difference, however, was not significant (Appendix K15). Chicks that previously had been fed a partially retinol deficient diet and fed a vitamin A-free diet from 7 weeks onward had serum IgM concentrations that

Table 14. Serum IgM concentrations of chicks as influenced by dietary retinol and retinoic acid supplementation before and 8 days after second vaccination with Newcastle disease virus–La Sota[a]

| Vitamin A source | ug/g diet | N[b] | Serum IgM (mg/ml) | | | |
|---|---|---|---|---|---|---|
| | | | Days after vitamin A withdrawal | | | |
| | | | 0 | 2 | 4 | 6 |
| Retinol | 2.0 | 4 | 1.69 | ND[c] | 2.94 | ND |
| | 0.2 | 4 | 2.28 | ND | 5.18 | ND |
| Retinoic acid | 2.0 | 4 | 2.33 | 4.11 | 3.87 | 5.19 |
| | 0.2 | 4 | | 4.28 | 4.52 | 5.62 |
| | 0 | 4 | | 4.59 | 6.89 | 8.74 |
| C.V.[d] | | | 10 | 17 | 29 | 37 |

Level of significance

| | | | | | | |
|---|---|---|---|---|---|---|
| Treatments | | | .0043 | NS[e] | .01 | .125 |
| Retinol 2 vs. retinoic acid 2 | | | .0025 | ND | NS | ND |
| Retinol 2 vs. 0.2 | | | .0043 | ND | .032 | ND |
| Retinoic acid 2 vs. 0.2 | | | | NS | NS | NS |
| Retinoic acid 2 vs. 0 | | | | NS | .006 | .064 |

[a]Vaccinations were given 2, 4, or 6 days after vitamin A was withdrawn from diets.

[b]N=Number of replicates.

[c]Not determined.

[d]Coefficient of variation.

[e]No significant difference among treatment means.

were significantly (p<.032) greater than those of retinol controls after

revaccination. Chicks that consumed diets supplemented with 0.2 ug

retinoic acid/g of diet had slightly greater average IgM concentrations

than retinoic acid controls. However, this difference was not significant

regardless of time of vaccination (Appendix K14-16). Average serum IgM

concentrations of totally RA deficient chicks were greater than RA

controls, with significant differences observed in chicks vaccinated 4 and

6 days after RA was removed from their diets.

After the second administration of antigen, an increase in serum IgA

concentrations of all chicks occurred (Table 15). As was observed with

IgG and IgM, serum IgA concentrations remained significantly (p<.037)

greater in chicks fed 2 ug RA/g of diet than in chicks fed 2 ug retinol/g

of diet after secondary vaccination (Appendix K15). Serum IgA concentra-

tions were significantly (p<.047) greater in chicks fed a retinol defi-

cient diet than a retinol adequate diet (Appendix K15). Unlike serum IgG

and IgM, IgA concentrations in serum obtained from vitamin A deficient

chicks previously fed 2 ug RA/g of diet showed no consistent pattern of

response to the different dietary treatments at any time of vaccination.

Serum IgA concentrations in chicks partially deficient in dietary RA were

not significantly different from those of RA controls (Appendix K14-16).

Chicks totally deficient in RA vaccinated 2 or 6 days after vitamin A was

removed from their diets, showed decreases in IgA concentrations as

compared with controls. A significant difference occurred between chicks

fed 0 and 2 ug RA/g of diet only on Day 2 of vaccination. In this

Table 15. Serum IgA concentration of chicks as influenced by dietary retinol and retinoic acid supplementation before and 8 days after second vaccination with Newcastle disease virus-La Sota[a]

| Vitamin A source | ug/g diet | N[b] | Serum IgA (mg/ml) Days after vitamin A withdrawal | | | |
|---|---|---|---|---|---|---|
| | | | 0 | 2 | 4 | 6 |
| Retinol | 2.0 | 4 | .388 | ND[c] | 1.47 | ND |
| | 0.2 | 4 | .722 | ND | 2.71 | ND |
| Retinoic acid | 2.0 | 4 | .855 | 2.93 | 2.78 | 2.84 |
| | 0.2 | 4 | | 2.64 | 2.84 | 3.33 |
| | 0 | 4 | | 1.41 | 2.92 | 2.16 |
| C.V.[d] | | | 28 | 40 | 32 | 41 |

| Level of significance | | | | |
|---|---|---|---|---|
| Treatments | .016 | .104 | .114 | NS[e] |
| Retinol 2 vs. retinoic acid 2 | .006 | ND | .037 | ND |
| Retinol 2 vs. 0.2 | .032 | ND | .047 | ND |
| Retinoic acid 2 vs. 0.2 | | NS | NS | NS |
| Retinoic acid 2 vs. 0 | | .048 | NS | NS |

[a]Vaccinations were given 2, 4, or 6 days after vitamin A was withdrawn from diets.

[b]N=Number of replicates.

[c]Not determined.

[d]Coefficient of variation.

[e]No significant difference among treatment means.

instance, serum IgA of the chicks fed 2 ug RA/g of diet was significantly (p<.048) greater than those of chicks fed no retinoic acid.

Serum hemagglutination inhibition antibody titers to NDV, expressed as geometric mean titers (GMT), were determined before final diet changes were made and at 8 days after chicks received their second vaccination of NDV. The results are presented in Table 16. After the second vaccination with NDV, antibody titers were three- to six-fold greater than those observed at 7 weeks of age (Day 0). Serum antibody titers were significantly (p<.027) greater in chicks fed diets supplemented with 2 ug RA/g of diet than in chicks fed 2 ug retinol/g of diet (Appendix K15). Despite the relatively high concentrations of serum IgG and IgM observed in vitamin A deficient chicks, serum antibody titers to NDV were significantly less in vitamin A deficient chicks than in vitamin A adequate controls. A significant decrease in antibody titer was observed whether chicks were fed diets totally or partially deficient in vitamin A or whether the vitamin A deficient chicks had previously been fed 2 ug RA or 0.2 ug retinol/g of diet (Appendix K14-16). Time of vaccination after vitamin A withdrawal did not affect the antibody titer response.

Chicken bile contains high concentrations of IgA as compared with other tissues. It was of interest, therefore, to assess changes in IgA concentrations of the bile in response to dietary vitamin A supplementation after exposure to antigen. Changes, in dry matter and total protein content of bile, related to dietary affects, after vaccination, were also examined.

Table 16. Serum hemagglutination inhibition titers of chicks as influenced by dietary retinol and retinoic acid supplementation before and 8 days after second vaccination with Newcastle disease virus-La Sota[a]

| Vitamin A source | ug/g diet | N[c] | Geometric mean titer[b] | | | |
|---|---|---|---|---|---|---|
| | | | Days after vitamin A withdrawal | | | |
| | | | 0 | 2 | 4 | 6 |
| Retinol | 2.0 | 4 | 27 | ND[d] | 661 | ND |
| | 0.2 | 4 | 22 | ND | 341 | ND |
| Retinoic acid | 2.0 | 4 | 22 | 879 | 1078 | 1003 |
| | 0.2 | 4 | | 458 | 618 | 396 |
| | 0 | 4 | | 450 | 377 | 305 |
| C.V.[e] | | | 34 | 19 | 39 | 44 |
| Level of significance | | | | | | |
| Treatments | | | NS[f] | .0005 | .0043 | .0063 |
| Retinol 2 vs. retinoic acid 2 | | | NS | ND | .027 | ND |
| Retinol 2 vs. 0.2 | | | NS | ND | .08 | ND |
| Retinoic acid 2 vs. 0.2 | | | | .001 | .017 | .007 |
| Retinoic acid 2 vs. 0 | | | | .001 | .001 | .003 |

[a]Vaccinations were given 2, 4, or 6 days after vitamin A was withdrawn from diets.

[b]Geometric mean titers (GMT) represents the reciprocal of the greatest dilution of serum showing inhibition of agglutination with antigen.

[c]N=Number of replicates.

[d]Not determined.

[e]Coefficient of variation.

[f]No significant difference among treatment means.

Concentrations of total protein per ml of bile of chicks, as determined before and after second vaccination with NDV, are presented in Table 17. The concentration of total protein in bile increased after vaccination, regardless of dietary treatment. Biliary protein concentrations varied greatly among chicks within the same treatment group (Appendix K17-20). As a result, no significant differences in total biliary protein were detected among the treatment groups vaccinated on Days 2 and 4 after final diet changes were made. However, chicks fed 0 or 0.2 ug RA/g of diet, and vaccinated 6 days after final diet changes were made, had significantly ($p < .001$) lower protein concentrations in bile than chicks fed 2 ug RA/g of diet.

The percentage of dry matter in bile samples taken directly from gall bladders of chicks before and after revaccination with NDV are presented in Table 18 (Appendix K17-20). The percent dry matter in bile was the same (26%) in all treatment groups before final diet changes were made (Day 0). The percentage of biliary dry matter increased 1 to 5% in RA and retinol control chicks after vaccination. A total deficiency of dietary retinol or RA significantly decreased dry matter content of bile obtained from chicks on either Day 2, 4, or 6 as compared with retinol and RA controls, respectively. In comparison with chicks fed 2 ug RA/g of diet, a significant ($p < .028$) decrease in biliary dry matter occurred in chicks fed 0.2 ug RA/g of diet and vaccinated 2 days after the vitamin A was removed from their diets.

Before the second administration of antigen (Day 0), the concentration of IgA in bile was significantly ($p < .027$) greater in chicks fed diets

Table 17. Total biliary protein of chicks as influenced by dietary retinol and retinoic acid supplementation before and 8 days after second vaccination with Newcastle disease virus-La Sota[a]

| Vitamin A source | ug/g diet | N[b] | Total biliary protein (mg/ml) Days after vitamin A withdrawal | | | |
|---|---|---|---|---|---|---|
| | | | 0 | 2 | 4 | 6 |
| Retinol | 2.0 | 4 | 34 | ND[c] | 60 | ND |
| | 0.2 | 4 | 31 | ND | 45 | ND |
| Retinoic acid | 2.0 | 4 | 33 | 93 | 47 | 63 |
| | 0.2 | 4 | | 74 | 71 | 39 |
| | 0 | 4 | | 66 | 69 | 44 |
| C.V.[d] | | | 12 | 27 | 34 | 12 |

| Level of significance | | | | |
|---|---|---|---|---|
| Treatments | NS[e] | NS | NS | .0007 |
| Retinol 2 vs. retinoic acid 2 | NS | | NS | ND |
| Retinol 2 vs. 0.2 | NS | | NS | ND |
| Retinoic acid 2 vs. 0.2 | | NS | NS | .001 |
| Retinoic acid 2 vs. 0 | | NS | NS | .001 |

[a]Vaccinations were given 2, 4, or 6 days after vitamin A was withdrawn from diets.

[b]N=Number of replicates.

[c]Not determined.

[d]Coefficient of variation.

[e]No significant difference among treatment means.

Table 18. Percentage of dry matter in bile of chicks as influenced by dietary retinol and retinoic acid supplementation before and 8 days after second vaccination with Newcastle disease virus-La Sota[a]

| Vitamin A source | ug/g diet | N[b] | Biliary dry matter (%) Days after vitamin A withdrawal | | | |
|---|---|---|---|---|---|---|
| | | | 0 | 2 | 4 | 6 |
| Retinol | 2.0 | 4 | 26 | ND[c] | 27 | ND |
| | 0.2 | 4 | 26 | ND | 25 | ND |
| Retinoic acid | 2.0 | 4 | 26 | 28 | 27 | 31 |
| | 0.2 | 4 | | 26 | 27 | 29 |
| | 0 | 4 | | 26 | 25 | 27 |
| C.V.[d] | | 3 | 5 | 5 | 6 | 6 |

| Level of significance | | | | |
|---|---|---|---|---|
| Treatments | NS[e] | .067 | .076 | .046 |
| Retinol 2 vs. retinoic acid 2 | NS | ND | NS | ND |
| Retinol 2 vs. 0.2 | NS | ND | .078 | ND |
| Retinoic acid 2 vs. 0.2 | | .028 | NS | NS |
| Retinoic acid 2 vs. 0 | | .081 | .027 | .016 |

[a]Vaccinations were given 2, 4, or 6 days after vitamin A was withdrawn from diets.

[b]N=Number of replicates.

[c]Not determined.

[d]Coefficient of variation.

[e]No significant difference among treatment means.

supplemented with 2 ug RA/g of diet as compared with chicks fed 2 ug

retinol/g of diet (Table 19, Appendix K17). After exposure to NDV 4 days

after final diet changes were made, the biliary IgA concentration in-

creased 61% in retinoic acid control chicks and 43% in retinol control

chicks. A single degree of freedom comparison, made after vaccination on

Day 4, indicated that biliary IgA concentrations were significantly

$(p<.012)$ greater in chicks fed 2 ug RA/g of diet than in chicks fed 2 ug

retinol/g of diet. No increase in IgA of bile, in response to vaccina-

tion, occurred in retinol deficient chicks fed a vitamin A-free diet as

compared with biliary IgA concentrations observed before vaccination.

Since biliary IgA concentrations increased after vaccination in chicks fed

retinol control diets but did not increase in chicks fed a retinol

deficient diet, a significant $(p<.049)$ decrease in IgA concentration of

bile from retinol deficient chicks as compared with retinol controls

occurred after vaccination (Appendix K19). When chicks previously fed 2

ug RA/g of diet were fed diets containing 0.2 ug RA/g of diet, the average

IgA concentration of bile increased after vaccination but remained lower

than that of the RA controls. The differences between partially deficient

and adequate RA chicks were significant on Day 4 and 6 (Appendix K19-20).

Biliary IgA concentrations decreased after vaccination of chicks fed 0 ug

RA/g of diet. This resulted in significantly $(p<.001)$ lower IgA concen-

trations of bile in chicks fed no RA as compared with those fed 2 ug RA/g

of diet. A significant decrease in biliary IgA in totally deficient

chicks occurred regardless at which time the chicks were vaccinated after

the onset of the deficiency (Appendix K18-20).

Table 19. Biliary IgA concentrations of chicks as influenced by dietary retinol and retinoic acid supplementation before and 8 days after second vaccination with Newcastle disease virus-La Sota[a]

| Vitamin A source | ug/g diet | N[b] | Biliary IgA (mg/ml) Days after vitamin A withdrawal | | | |
| | | | 0 | 2 | 4 | 6 |
|---|---|---|---|---|---|---|
| Retinol | 2.0 | 4 | 16 | ND[c] | 23 | ND |
| | 0.2 | 4 | 17 | ND | 17 | ND |
| Retinoic acid | 2.0 | 4 | 20 | 30 | 32 | 41 |
| | 0.2 | 4 | | 26 | 25 | 21 |
| | 0 | 4 | | 14 | 19 | 17 |
| C.V.[d] | | | 12 | 18 | 20 | 25 |

Level of significance

| | | | | | | |
|---|---|---|---|---|---|---|
| Treatments | | | .072 | .001 | .002 | .001 |
| Retinol 2 vs. retinoic acid 2 | | | .027 | ND | .012 | ND |
| Retinol 2 vs. 0.2 | | | NS[e] | ND | .049 | ND |
| Retinoic acid 2 vs. 0.2 | | | | NS | .045 | .002 |
| Retinoic acid 2 vs. 0 | | | | .001 | .001 | .001 |

[a]Vaccinations were given 2, 4, or 6 days after vitamin A was withdrawn from diets.

[b]N=Number of replicates.

[c]Not determined.

[d]Coefficient of variation.

[e]No significant difference among treatment means.

The portion of intestine extending 10 cm above to 10 cm below the yolk stalk were removed from chicks of the different dietary treatment groups before and after secondary vaccination with NDV. Data describing weights and total protein concentrations of these tissues are presented in Table 20. The weight of intestinal samples removed from retinol deficient and RA control chicks did not differ significantly from retinol control chicks at 7 weeks of age (Day 0) (Appendix K21). However, the average intestinal weight of samples taken after vaccination were significantly ($p < .0142$) less for chicks fed diets containing 2 ug RA/g of diet (5.52 g) than for chicks fed the same amount of vitamin A in the form of retinol (6.30 g) (Appendix K23). Statistical evaluation of data obtained from chicks vaccinated 2 days after final diet changes were made indicated that vitamin A concentration had no significant effect on intestine weight (Appendix K22). Chicks previously fed diets supplemented with 0.2 ug retinol or 2 ug RA/g of diet and vaccinated 4 or 6 days after consuming a vitamin A-free diet had significantly less intestine weights than their respective vitamin A controls. Intestine weights of chicks fed a partial concentration of required dietary RA were not significantly different than those of RA control chicks.

Total protein per g of intestine was not affected significantly by diet treatment (Appendix K21-24). After the second vaccination with NDV, which included both local and systemic applications of antigen, average intestinal total protein concentrations did not change appreciably from prevaccination concentrations.

Table 20. Weight and total protein concentration of intestine from chicks before and 8 days after second vaccination with Newcastle disease virus-La Sota[a]

| Vitamin A source | ug/g diet | N[b] | Days after vitamin A withdrawal | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | 0 | | 2 | | 4 | | 6 | |
| | | | Tissue wt (g) | Total protein mg/g tissue | Tissue wt (g) | Total protein mg/g tissue | Tissue wt (g) | Total protein mg/g tissue | Tissue wt (g) | Total protein mg/g tissue |
| Retinol | 2.0 | 4 | 6.22 | 63.78 | ND[c] | ND | 6.30 | 62.73 | ND | ND |
| | 0.2 | 4 | 5.66 | 68.35 | ND | ND | 5.61 | 64.57 | ND | ND |
| Retinoic acid | 2.0 | 4 | 5.57 | 68.33 | 6.22 | 63.80 | 5.52 | 65.79 | 5.28 | 66.26 |
| | 0.2 | 4 | | | 5.66 | 68.33 | 5.54 | 63.30 | 5.07 | 67.41 |
| | 0 | 4 | | | 5.57 | 68.34 | 4.54 | 69.84 | 4.08 | 56.82 |
| C.V.[d] | | | 9.16 | 12.42 | 9.16 | 12.45 | 7.25 | 12.78 | 7.49 | 12.33 |

Level of significance

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Treatment | | | NS[e] | NS | NS | NS | .0004 | NS | .0025 | NS |
| Retinol 2 vs. retinoic acid 2 | | | NS | NS | NS | ND | .0142 | NS | ND | ND |
| Retinol 2 vs. 0.2 | | | NS | NS | ND | ND | .0268 | NS | ND | ND |
| Retinoic acid 2 vs. 0.2 | | | | | NS | NS | NS | NS | NS | NS |
| Retinoic acid 2 vs. 0 | | | | | NS | NS | .0033 | NS | .001 | NS |

[a]Vaccinations were administered 2, 4, or 6 days after vitamin A was withdrawn from diets.

[b]N=Number of replicates.

[c]Not determined.

[d]Coefficient of variation.

[e]No significant difference between treatment means.

The importance of vitamin A in local immune function was assessed by determining the concentration of immunoglobulins in intestine of chicks fed diets supplemented with various concentrations of vitamin A. The concentrations of immunoglobulins in intestine are presented in Tables 21 through 23 as mg/g of tissue. The concentration of IgG in intestinal samples obtained from chicks before and after revaccination with NDV are presented in Table 21. Not all of the vitamin A control groups exhibited an increase in intestinal IgG concentrations, after vaccination, above the concentration that occurred at 7 weeks of age (Day 0). This indicated that IgG concentrations in intestine did not increase in response to second vaccination with NDV as was observed for serum IgG concentrations. However, the pattern of response to dietary treatment was similar for intestinal IgG concentration as for serum IgG concentrations. At 7 weeks of age, the IgG concentration of intestine was significantly greater in retinol deficient and RA control chicks than in retinol controls (Appendix K21). After vaccination, average IgG concentrations of intestine from RA control chicks remained significantly ($p < .100$) greater than in the intestine from retinol controls (Appendix K23). At each time of vaccination, intestinal IgG concentrations were significantly greater in chicks fed a vitamin A-free diet in the form of either retinol or RA as compared with their respective vitamin A controls (Appendix K22-24). Chicks fed diets containing 0.2 ug RA/g of diet had average intestinal IgG concentrations that were numerically greater than chicks fed 2 ug RA/g of diet but the difference was not significant.

Table 21. Intestinal IgG concentrations of chicks as influenced by dietary retinol and retinoic acid supplementation before and 8 days after second vaccination with Newcastle disease virus-La Sota[a]

| Vitamin A source | ug/g diet | N[b] | Intestinal IgG (mg/g tissue) Days after vitamin A withdrawal | | | |
|---|---|---|---|---|---|---|
| | | | 0 | 2 | 4 | 6 |
| Retinol | 2.0 | 4 | .454 | ND[c] | .388 | ND |
| | 0.2 | 4 | .547 | ND | 1.00 | ND |
| Retinoic acid | 2.0 | 4 | .593 | .634 | .728 | .575 |
| | 0.2 | 4 | | .906 | .853 | 1.05 |
| | 0 | 4 | | 1.18 | 2.04 | 1.96 |
| C.V.[d] | | | 14 | 29 | 28 | 58 |
| Level of significance | | | | | | |
| Treatments | | | .066 | .05 | .0001 | .066 |
| Retinol 2 vs. retinoic acid 2 | | | .025 | ND | .100 | ND |
| Retinol 2 vs. 0.2 | | | .100 | ND | .0068 | ND |
| Retinoic acid 2 vs. 0.2 | | | | NS[e] | NS | NS |
| Retinoic acid 2 vs. 0 | | | | .018 | .0001 | .025 |

[a]Vaccinations were given 2, 4, or 6 days after vitamin A was withdrawn from diets.

[b]N=Number of replicates.

[c]Not determined.

[d]Coefficient of variation.

[e]No significant difference among treatment means.

Table 22. Intestinal IgM concentration of chicks as influenced by dietary retinol and retinoic acid supplementation before and 8 days after second vaccination with Newcastle disease virus-La Sota[a]

| Vitamin A source | ug/g diet | N[b] | Intestinal IgM (mg/g tissue) Days after vitamin A withdrawal | | | |
|---|---|---|---|---|---|---|
| | | | 0 | 2 | 4 | 6 |
| Retinol | 2.0 | 4 | .109 | ND[c] | .147 | ND |
| | 0.2 | 4 | .126 | ND | .326 | ND |
| Retinoic acid | 2.0 | 4 | .115 | .167 | .294 | .235 |
| | 0.2 | 4 | | .163 | .279 | .339 |
| | 0 | 4 | | .235 | .627 | .717 |
| C.V.[d] | | | 11 | 27 | 65 | 53 |
| Level of significance | | | | | | |
| Treatments | | | .213 | .129 | .0757 | .034 |
| Retinol 2 vs. retinoic acid 2 | | | NS[e] | ND | NS | ND |
| Retinol 2 vs. 0.2 | | | .092 | ND | NS | ND |
| Retinoic acid 2 vs. 0.2 | | | | NS | NS | NS |
| Retinoic acid 2 vs. 0 | | | | .087 | .0474 | .015 |

[a]Vaccinations were given 2, 4, or 6 days after vitamin A was withdrawn from diets.

[b]N=Number of replicates.

[c]Not determined.

[d]Coefficient of variation.

[e]No significant difference among treatment means.

Table 23. Intestinal IgA concentration of chicks as influenced by dietary retinol and retinoic acid supplementation before and 8 days after second vaccination with Newcastle disease virus–La Sota[a]

| Vitamin A source | ug/g diet | N[b] | Intestinal IgA (mg/g tissue) Days after vitamin A withdrawal | | | |
|---|---|---|---|---|---|---|
| | | | 0 | 2 | 4 | 6 |
| Retinol | 2.0 | 4 | .263 | ND[c] | .194 | ND |
| | 0.2 | 4 | .292 | ND | .222 | ND |
| Retinoic acid | 2.0 | 4 | .267 | .197 | .271 | .159 |
| | 0.2 | 4 | | .189 | .215 | .125 |
| | 0 | 4 | | .165 | .222 | .109 |
| C.V.[d] | | | 22 | 38 | 42 | 22 |
| Level of significance | | | | | | |
| Treatments | | | NS[e] | NS | NS | .087 |
| Retinol 2 vs. retinoic acid 2 | | | NS | ND | NS | ND |
| Retinol 2 vs. 0.2 | | | NS | ND | NS | ND |
| Retinoic acid 2 vs. 0.2 | | | | NS | NS | NS |
| Retinoic acid 2 vs. 0 | | | | NS | NS | .034 |

[a]Vaccinations were given 2, 4, or 6 days after vitamin A was withdrawn from diets.

[b]N=Number of replicates.

[c]Not determined.

[d]Coefficient of variation.

[e]No significant difference among treatment means.

IgM contents of intestinal samples obtained from chicks before and after vaccination are presented in Table 22. Intestinal IgM concentrations, when expressed as mg of IgM/g of tissue, paralleled the response of serum IgM concentration to different dietary vitamin A regimes. The data indicate a small increase in intestinal IgM content in all chicks after vaccination with NDV, irrespective of dietary treatment. Before vaccination (Day 0), average intestinal IgM concentrations were greater in retinol deficient and RA control chicks than in retinol controls. Average intestinal IgM concentrations remained greater in RA control chicks and retinol deficient chicks than retinol control chicks after vaccination, but the differences were not significant (Appendix K21). There were no significant differences in intestinal IgM concentrations of chicks fed 0.2 and those fed 2 ug retinoic acid/g of diet. Chicks that consumed diets totally deficient in RA had significantly greater intestinal IgM concentrations than did the retinoic acid control chicks, regardless of vaccination (Appendix K22-24).

Milligrams of IgA per g of intestine, as influenced by dietary vitamin A supplementation before and after vaccination, are presented in Table 23 (Appendix K21-24). No increase in intestinal IgA concentration above that observed before vaccination occurred as a result of vaccination, irrespective of dietary treatment. There were no differences in intestinal IgA concentrations of the three dietary treatment groups before vaccination (Day 0). When determined 8 days after revaccination (Day 4), average intestinal IgA concentrations were greater in chicks fed diets supplemented with 2 ug retinoic acid/g of diet than in chicks fed the

equivalent amount of vitamin A in the form of retinol. The difference

between the two groups, however, was not significant. There was a

consistent decrease in the average intestinal IgA concentration of chicks

fed 0 and 0.2 ug RA/g of diet as compared with the intestinal IgA

concentrations of chicks fed 2 ug retinoic acid/g of diet. This decrease

was observed at each vaccination time. A significant ($p < .034$) difference

between RA deficient chicks and RA control chicks occurred only in chicks

that consumed no dietary RA for 6 days before vaccination. Intestinal IgA

concentrations in retinol deficient and retinol adequate chicks, however,

did not follow the same trend. Retinol deficient chicks fed a vitamin

A-free diet for 4 days before vaccination had a greater average intestinal

IgA concentration than retinol control chicks but this difference was not

statistically significant.

Tracheal samples removed from chicks before and after vaccination

were weighed and the amount of total protein in each g of tissue was

determined. These data are presented in Table 24. Statistical analysis

of tissue weight data, obtained from the initial three dietary treatment

groups at 7 weeks of age (Day 0), revealed no significant treatment

effects (Appendix K25). Single degree of freedom comparisons made on Day

4 of vaccination confirmed that RA at a concentration of 2 ug/g of diet

was adequate in supporting tracheal weight equal to that obtained from

chicks fed 2 ug retinol/g of diet (Appendix K27). However, after

vaccination, absolute tracheal weight was strongly affected by the

concentration of vitamin A in the diet. Chicks consuming 0 or 0.2 ug

vitamin A/g of diet had smaller trachea weights than chicks fed an

Table 24. Weight and total protein concentration of trachea from chicks before and 8 days after second vaccination with Newcastle disease virus–La Sota[a]

| Vitamin A source | ug/g diet | N[b] | Days after vitamin A withdrawal | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | 0 | | 2 | | 4 | | 6 | |
| | | | Tissue wt (g) | Total protein mg/g tissue | Tissue wt (g) | Total protein mg/g tissue | Tissue wt (g) | Total protein mg/g tissue | Tissue wt (g) | Total protein mg/g tissue |
| Retinol | 2.0 | 4 | 1.58 | 28 | ND[c] | ND | 2.20 | 29 | ND | ND |
| | 0.2 | 4 | 1.65 | 29 | ND | ND | 2.01 | 27 | ND | ND |
| Retinoic acid | 2.0 | 4 | 1.53 | 29 | 2.15 | 18 | 2.14 | 27 | 2.19 | 26 |
| | 0.2 | 4 | | | 1.92 | 19 | 1.88 | 28 | 2.12 | 25 |
| | 0 | 4 | | | 1.83 | 19 | 1.64 | 33 | 1.92 | 25 |
| c.v.[d] | | | 7.2 | 8.1 | 6.4 | 6.4 | 7.4 | 9.2 | 11 | 9.6 |

| Level of significance | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Treatment | NS[e] | NS | | .016 | NS | | .0006 | .043 | NS | NS |
| Retinol 2 vs. retinoic acid 2 | NS | NS | | ND | | | NS | NS | ND | ND |
| Retinol 2 vs. 0.2 | NS | NS | | ND | | | .087 | NS | ND | ND |
| Retinoic acid 2 vs. 0.2 | | | | .032 | NS | | .028 | NS | NS | NS |
| Retinoic acid 2 vs. 0 | | | | .006 | NS | | .0002 | .009 | NS | NS |

[a]Vaccinations were administered 2, 4, or 6 days after vitamin A was withdrawn from diets.

[b]N=Number of replicates.

[c]Not determined.

[d]Coefficient of variation.

[e]No significant difference between treatment means.

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adequate concentration of retinol or RA. The more severe the dietary

deficiency, the less the tissue weighed. Statistically significant

differences, however, occurred only on Days 2 and 4, but the trend towards

decreased tissue weight with decreasing dietary vitamin A remained on Day

4.

In order to express immunoglobulin concentration as a function of

total tissue protein, the total protein concentration of each tracheal

sample was determined. There were no significant differences among

treatment groups in total protein concentration of tracheal samples

(Appendix K25-28). This was true except for a significant ($p < .009$)

increase in total tracheal protein which occurred in chicks fed 0 ug RA/g

of diet as compared with chicks fed 2 ug RA/g of diet. This significant

difference was observed only when these diets were fed for 4 days before

chicks were given a second vaccination of NDV.

All three types of immunoglobulins were detected in supernatants of

homogenized tracheal samples. The concentration of immunoglobulin

expressed as mg/g of tissue is shown in Tables 25 through 27. Averaged

over all treatments, IgG concentrations in trachea were less before

vaccination than after vaccination with NDV (Table 25). A pattern of

response to dietary treatments similar to that observed for serum and

intestinal IgG was observed on Day 0 in tracheal tissue. This included a

significant increase in IgG concentration in both retinol deficient (0.2

ug/g of diet) and RA controls (2 ug/g of diet) as compared with chicks fed

retinol control diets (2 ug/g of diet) (Appendix K25). When chicks were

vaccinated 4 days after final diet change was made, the trend of treatment

Table 25. Tracheal IgG concentrations of chicks as influenced by dietary retinol and retinoic acid supplementation before and 8 days after second vaccination with Newcastle disease virus-La Sota[a]

| Vitamin A source | ug/g diet | N[b] | Tracheal IgG (mg/g tissue) Days after vitamin A withdrawal | | | |
|---|---|---|---|---|---|---|
| | | | 0 | 2 | 4 | 6 |
| Retinol | 2.0 | 4 | .463 | ND[c] | .560 | ND |
| | 0.2 | 4 | .522 | ND | .740 | ND |
| Retinoic acid | 2.0 | 4 | .549 | .770 | .700 | .338 |
| | 0.2 | 4 | | .706 | .675 | .535 |
| | 0 | 4 | | .821 | 1.34 | .765 |
| C.V.[d] | | | 9 | 22 | 25 | 25 |

Level of significance

| | 0 | 2 | 4 | 6 | |
|---|---|---|---|---|---|
| Treatments | .05 | .05 | NS[e] | .0005 | .005 |
| Retinol 2 vs. retinoic acid 2 | .02 | ND | NS | ND | |
| Retinol 2 vs. 0.2 | .09 | ND | NS | ND | |
| Retinoic acid 2 vs. 0.2 | | NS | NS | .065 | |
| Retinoic acid 2 vs. 0 | | | .0004 | .002 | |

[a]Vaccinations were given 2, 4, or 6 days after vitamin A was withdrawn from diets.

[b]N=Number of replicates.

[c]Not determined.

[d]Coefficient of variation.

[e]No significant difference among treatment means.

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Table 26. Tracheal IgM concentration of chicks as influenced by dietary retinol and retinoic acid supplementation before and 8 days after second vaccination with Newcastle disease virus-La Sota[a]

| Vitamin A source | ug/g diet | N[b] | Tracheal IgM (mg/g tissue) Days after vitamin A withdrawal | | | |
|---|---|---|---|---|---|---|
| | | | 0 | 2 | 4 | 6 |
| Retinol | 2.0 | 4 | .169 | ND[c] | .248 | ND |
| | 0.2 | 4 | .193 | ND | .379 | ND |
| Retinoic acid | 2.0 | 4 | .189 | .250 | .252 | .218 |
| | 0.2 | 4 | | .270 | .261 | .249 |
| | 0 | 4 | | .249 | .429 | .305 |
| C.V.[d] | | | 11 | 16 | 28 | 36 |
| Level of significance | | | | | | |
| Treatments | | | NS[e] | NS | .028 | NS |
| Retinol 2 vs. retinoic acid 2 | | | NS | ND | NS | ND |
| Retinol 2 vs. 0.2 | | | NS | ND | .05 | ND |
| Retinoic acid 2 vs. 0.2 | | | | NS | NS | NS |
| Retinoic acid 2 vs. 0 | | | | NS | .01 | NS |

[a]Vaccinations were given 2, 4, or 6 days after vitamin A was withdrawn from diets.

[b]N=Number of replicates.

[c]Not determined.

[d]Coefficient of variation.

[e]No significant difference among treatment means.

Table 27. Tracheal IgA concentration of chicks as influenced by dietary retinol and retinoic acid supplementation before and 8 days after second vaccination with Newcastle disease virus-La Sota[a]

| Vitamin A source | ug/g diet | N[b] | Tracheal IgA (mg/g tissue) Days after vitamin A withdrawal | | | |
|---|---|---|---|---|---|---|
| | | | 0 | 2 | 4 | 6 |
| Retinol | 2.0 | 4 | .191 | ND[c] | .232 | ND |
| | 0.2 | 4 | .225 | ND | .183 | ND |
| Retinoic acid | 2.0 | 4 | .241 | .101 | .193 | .161 |
| | 0.2 | 4 | | .063 | .101 | .134 |
| | 0 | 4 | | .051 | .335 | .101 |
| c.v.[d] | | | 38 | 38 | 47 | 44 |

Level of significance

| | | | | | | |
|---|---|---|---|---|---|---|
| Treatments | | | NS[e] | .07 | .05 | NS |
| Retinol 2 vs. retinoic acid 2 | | | NS | ND | NS | ND |
| Retinol 2 vs. 0.2 | | | NS | ND | NS | ND |
| Retinoic acid 2 vs. 0.2 | | | | .08 | NS | NS |
| Retinoic acid 2 vs. 0 | | | | .03 | .06 | NS |

[a]Vaccinations were given 2, 4, or 6 days after vitamin A was withdrawn from diets.

[b]N=Number of replicates.

[c]Not determined.

[d]Coefficient of variation.

[e]No significant difference among treatment means.

effects remained the same, but single degree of freedom comparisons failed to detect significant differences between treatments. IgG concentrations of trachea from chicks fed a diet containing 0.2 ug RA/g of diet, for 2 or 6 days before vaccination, did not differ significantly from those of chicks that continued to receive diets supplemented with 2 ug RA/g of diet. When 6 days passed before the partially vitamin A deficient chicks were vaccinated, a significant ($p < .065$) increase in tracheal IgG concentration occurred as compared with RA adequate controls. A total lack of dietary RA resulted in a greater average tracheal IgG concentration as compared with chicks fed 2 ug RA/g of diet. This diet effect occurred regardless of time of vaccination, but significant differences were detected only on Days 4 and 6.

The concentration of IgM in tracheal tissue removed from chicks before and after revaccination with NDV are presented in Table 26. Low concentrations of IgM were detected in tracheal tissue obtained from chicks at 7 weeks of age (Day 0). This occurred because chicks had not received a second vaccination of NDV. After exposure to local and systemic application of antigen, IgM concentrations of trachea increased in all chicks, well above those attained at 7 weeks of age. Average concentrations of IgM in trachea were greater in RA control and retinol deficient chicks than retinol controls. These differences in IgM concentrations occurred both before (Day 0) and after vaccination (Day 4). Single degree of freedom comparisons did not yield significant differences among these three treatment groups on Day 0 (Appendix K25). However, a significant ($p < .05$) difference between chicks fed 2 ug and 0 ug retinol/g

of diet was observed on Day 4 (Appendix K27). Another significant (p<.01) single degree of freedom comparison made on Day 4, between chicks fed 2 and 0 ug RA/g of diet indicated a trend towards increased IgM concentrations in trachea of vitamin A deficient chicks as compared with controls. However, no significant diet effects were observed among chicks vaccinated 2 or 6 days after final diet changes were made (Appendix K26 and K28).

In response to exposure to NDV, IgA concentrations/g of trachea decreased from prevaccination concentrations, in all but two treatment groups (Table 27). This was an indication that tracheal tissue was not responding to antigenic exposure by increasing its IgA concentration appreciably. At 7 weeks of age, average tracheal IgA concentrations were greater in chicks fed retinol deficient and RA adequate diets than in retinol controls. This trend was similar to serum IgA concentrations. Diet effects at this time, however, were not significant (Appendix K25). After vaccination, there was a tendency for vitamin A-deficient chicks to have lower concentrations of IgA in trachea than chicks fed vitamin A control diets. Statistical evaluation of data at Day 2 indicated significantly lower tracheal IgA concentrations in chicks fed 0 and 0.2 ug RA/g of diet than in chicks fed 2 ug RA/g of diet (Appendix K26). If chicks were fed a vitamin A-deficient diet for 4 or 6 days before vaccination, their average tracheal IgA concentrations were lower than the respective vitamin A controls. This was true except for chicks fed 0 ug RA/g of diet and vaccinated 4 days after the diet was fed. These chicks had significantly (p<.06) greater tracheal IgA concentrations than chicks fed 2 ug RA/g of diet (Appendix K27-28).

DISCUSSION

Ataxia and incoordination were the first signs of vitamin A deficiency observed. The onset of deficiency occurred 13 days after day-old chicks were fed vitamin A-free diets. Unsteadiness was alleviated when the vitamin A-deficient chicks were fed diets supplemented with retinoic acid at a concentration of 2 ug/g of diet or retinol at a concentration of 2 or .2 ug/g of diet.

Chicks fed a retinol-low diet (.2 ug/g diet) after depletion of hepatic vitamin A stores, grew as well as chicks fed a diet supplemented with 2 ug of retinol/g of diet for a 2-week period. After 2 weeks of supplementation, the vitamin A deficient diet began to impair the growth of chicks. After 3 weeks of further supplementation, body weight of chicks fed .2 ug of retinol/g of diet averaged only 80% of the body weights of chicks fed 2 ug retinol/g of diet. At 7 weeks of age, 18.5 ug of retinol was detected per g of liver in chicks fed diets containing 2 ug retinol/g of diet. Chicks fed .2 ug of retinol/g of diet had only 2.89 ug of vitamin A/g of liver. Twelve days after the feeding of a vitamin A-free diet began, the liver vitamin A concentrations, of chicks previously fed diets containing the lower concentration of retinol (.2 ug/g of diet), fell to 1.27 ug/g of liver.

Retinoic acid has been shown to function as well as retinol in support of body weight gain of rats (Moore, 1976; Chole and Quick, 1978). Chicks in the present study that were fed a diet supplemented with 2 ug of RA/g of diet grew as well as chicks fed the same amount of vitamin A in the form of retinol. Growth was maintained in chicks fed 2 ug of RA/g of

diet despite the absence of any detectable liver vitamin A at 7 weeks of age.

After chicks fed 2 ug of RA/g of diet were changed to a diet devoid of vitamin A, a plateau in weight gain occurred. Ten, 12, and 14 days after the vitamin A-free dietary regimen began, body weights were significantly less when compared with chicks that remained on diets supplemented with RA. The impaired growth rate was accompanied by additional signs of vitamin A deficiency including ataxia, eye lesions, incoordination and ruffled plumage. In contrast, chicks that were fed 2 ug of RA/g of diet and were changed to a diet containing .2 ug of RA/g of diet grew as well, during the 10- to 14-day deprivation period, as chicks that remained on the adequate RA diet. These results are similar to those of Panda and Combs (1963) where average 8-week body weights of chicks fed suboptimal concentrations of vitamin A were 98% of controls.

The total protein concentration in serum of chicks in the present study were higher than concentrations reported for specific pathogen-free chicks of the same age (Gillette, 1980) but were similar to values observed in conventionally raised chicks (Morgan and Glick, 1972). From the time chicks were given a primary vaccination with NDV until 7 weeks of age, serum protein concentrations did not change dramatically. Total serum protein, after primary vaccination, was not affected by vitamin A status of the chicks.

There was a definite increase in total serum protein after the second vaccination with NDV. An increase occurred in all chicks, irrespective of dietary treatment. However, chicks previously fed 2 ug RA/g of diet and

vaccinated 4 days after vitamin A was removed from their diet had significantly greater serum protein concentrations than chicks that remained on the RA supplemented diet. The increase in serum immunoglobulin concentrations observed in vitamin A deficient chicks may have contributed to the increase in total protein observed in this particular group of chicks. In most cases, total serum protein concentrations did not differ significantly among treatment groups after secondary vaccination.

Chicks were given a primary vaccination of NDV at 2 weeks of age. The time at which the primary vaccination was given was dependent upon the concentration of maternally derived antibody to NDV present in serum of the young birds. Optimum immunization or antibody response in the chick is affected by the concentration of antibody received passively from their dams (Allan et al., 1978). The development of serum antibody in the young bird has been shown to be adversely affected by the presence of maternal antibody (Ewert and Eidson, 1977). In young chicks, maternal antibody may neutralize locally applied vaccine virus in the respiratory tract before the virus can establish an infection and stimulate additional antibody production (Ewert and Eidson, 1977). The concentration of passively acquired maternal antibody in the young chick generally declines at a constant rate and has a half-life of four and one-half days (Allan et al., 1978). The age at which the chick will respond satisfactory to NDV can therefore be estimated. Previous workers have estimated the age for successful vaccination of NDV based on serum hemagglutination inhibition titer (Allan et al., 1978). If the average geometric mean titer (GMT) of day-old chicks is greater than 128, there is not likely to be an active

immune response to immunization before the chicks are 21 days of age.

When chicks have hemagglutination titers of 63 or less at 1 day of age,

immunization at 14 days of age can be expected to be successful. Chicks

in the present study had an average GMT of 32 at 1 day of age. They were,

therefore, expected to be largely free of maternal antibody by 14 days of

age. The actual average GMT at 14 days of age was below 6. Fourteen days

of age was the time at which chicks were given a live dose of a B1 strain

of NDV intraocularly and intranasally. The average GMT, irrespective of

dietary treatment, increased only slightly after the primary vaccination.

The maternal antibody still present at the time of vaccination may have

been sufficient to inhibit a strong primary antibody response.

The time interval between the primary and secondary vaccination also

has been shown to affect the antibody response birds will have to NDV

vaccination (Allan et al., 1978). If the time interval is less than 21

days, the antibody produced by the first dose may interfere with the

multiplication of the second dose of virus. As the antibody response to

the first vaccination decreases with time, delaying of the second dose can

elicit a greater response (Allan et al., 1978). Therefore, chicks in the

present study were given a second dose of an attenuated La Sota strain of

NDV between 35 and 41 days after the first dose of NDV was given. In

contrast to the primary response, revaccination with the La Sota strain of

NDV, by intraocular, intranasal, and intramuscular routes, led to an

increase in antibody concentration far above that obtained after the

primary vaccination.

In the present study, chicks were revaccinated 2, 4, or 6 days after final diet changes were made. Final diet changes were made at 7 weeks of age. At this time, chicks previously fed .2 ug of retinol/g of diet were changed to a vitamin A-free diet, whereas chicks fed diets containing 2 ug retinol/g of diet remained on this diet. Chicks previously fed 2 ug of RA/g of diet were changed to 0 or .2 ug RA/g of diet. Some of the groups of chicks previously fed 2 ug of RA/g of diet remained on this diet and served as controls. In general, prolonging the deprivation period 2 or 4 additional days, before vaccination, did not appear to affect the results of the study. In most cases, 2 days of deprivation before the second dose of NDV was given was a sufficient period of time to produce differences among dietary treatment groups. Differences among treatment groups may not have been statistically significant at all sampling times. However, in most cases, a consistent pattern of response was observed.

An increase in hemagglutination inhibition precipitating serum antibody to NDV was detected both after the primary and secondary vaccinations. Serum antibody to NDV vaccination peaked 2 weeks after primary vaccination after which time the antibody response decreased. Eight days after the second dose of NDV was given, the GMT rose four to five fold from prevaccination titers. An increase in antibody titer after primary and secondary vaccination with NDV occurred in all chicks, regardless of dietary treatment. There was no significant difference in the concentration of serum antibody in response to the primary vaccination between vitamin A deficient and control chicks. However, antibody titers

after the second NDV vaccination were significantly lower in chicks fed vitamin A deficient diets as compared with controls.

A pronounced impairment of the secondary response to NDV clearly demonstrates that vitamin A deficiency has an inhibitory effect on the antibody response. Previous assessments of the capacity of animals and humans in a vitamin A deficient state to form humoral antibodies, as tested by challenge with viral or bacterial vaccines, has given variable results. For example, normal antibody responses to diptheria and tetanus vaccine have been reported in vitamin A deficient children (Kutty et al., 1981). Panda and Combs (1963), however, detected lower agglutinin responses to S. Pullorum challenge in chicks fed diets partly deficient in vitamin A as compared with controls. In the present study, both a total as well as a partial deficiency of dietary RA and retinol significantly decreased serum antibody response to NDV when compared with control chicks.

The mechanism whereby vitamin A deficiency impairs specific antibody response as assessed by hemagglutination inhibition assay has not been elucidated. Cooperation among T lymphocytes, B lymphocytes, and macrophages is required for optimum antibody response. Any defect in the ability of these cells to cooperate could result in impaired immune response. The number of circulating B lymphocytes is normal during vitamin A deficiency (Kutty et al., 1981); however, a decrease in T lymphocytes in serum of vitamin A deficient children has been reported (Bhaskaram and Reddy, 1975; Kutty et al., 1981). Nauss et al. (1979) reported that the mitogenic response of T lymphocytes was depressed in

vitamin A deficient rats. Vitamin A deficiency could alter membrane receptors thereby affecting lymphocyte proliferation. Glycoproteins are important cell-surface receptors and may comprise the recognition sites on the surface of lymphocytes (Gesner and Ginsberg, 1964). A direct role of vitamin A in glycoprotein synthesis has been demonstrated (DeLuca et al., 1970).

A great deal of evidence suggests that vitamin A enhances the functional capacities of phagocytic cells (Cohen and Cohen, 1973; Krishnan et al., 1976; Tengerdy and Brown, 1977). Because macrophages are involved in the induction of immune responses, regulatory effects of vitamin A on macrophage activity would be critical for optimum antibody production.

It is possible that the deleterious effects of vitamin A deficiency upon serum antibody production may be related to a direct effect on the synthesis and/or secretion of immunoglobulins. It has been demonstrated that vitamin A is associated with the synthesis of RNA and DNA (Zile et al., 1979). However, no deficiency of IgG or IgM was found in the serum of vitamin A deficient chicks in the present study. On the contrary, the serum concentration of immunoglobulins in vitamin A deficient chicks was higher than those of controls. During a 5-week period, in which chicks were partly deficient in vitamin A, serum immunoglobulins IgG, IgM, and IgA began to increase above those observed in vitamin A adequate control chicks. By the end of the 5-week period, all three classes of immuno-globulin were significantly greater in the vitamin A deficient chicks as compared with controls. Similarly, serum IgG and IgM concentrations were significantly greater in vitamin A deficient chicks than controls 8 days

after secondary vaccination with NDV. The response of serum IgA to secondary vaccination in vitamin A deficient chicks was not consistent. Serum IgA concentrations were greater in retinol deficient chicks after revaccination, but lower or similar amounts of IgA were detected in the serum of RA deficient chicks as compared with controls.

An explanation for increased immunoglobulin concentrations despite diminished antibody response is not clear. The immunoglobulin molecules that are secreted may be defective in quality. When antibody response is determined by hemagglutination inhibition assay, a decreased affinity for antigen in the quantitation plates may be occurring.

Several workers have observed high concentrations of IgG, IgM, and IgA in the serum of bursectomized chicks (Carey and Warner, 1964; Lerner et al., 1971; Granfors et al., 1982). The chicks, however, could not produce specific antibodies to a number of antigens used for immunization. These studies indicated that bursectomy caused production of qualitatively defective antibody. It has been suggested that increases in immuno-globulin concentrations in chickens infected with Marek's disease virus could be related to viral destruction of the bursa of Fabricius and other lymphoid tissues (Higgins and Calnek, 1975a). The increase in immuno-globulin concentration after secondary vaccination in vitamin A deficient chicks in the present study could also be attributed to damaged lymphoid tissue. This explanation is consistent with the finding that vitamin A deficiency produces a moderate loss of lymphocytes from thymus and bursa of Fabricius, while infection of vitamin A deficient chicks with NDV

causes a rapid subtotal or total loss of lymphocytes from these tissues (Bang et al., 1973).

A second contributing factor for increased immunoglobulin concentrations, in the presence of diminished antibody response, may be depressed T lymphocyte function. In protein-energy malnourished humans and in animals exhibiting reduced cell-mediated immunity, high concentrations of IgE have been observed (Kikkama et al., 1973). A depression of cell-mediated and T lymphocyte function observed during vitamin A deficiency (Nauss et al., 1979) may be associated with a loss of T cell suppression and control of B lymphocytes leading, in turn, to increased nonspecific immunoglobulin synthesis.

The increased immunoglobulin concentrations may, however, represent antibodies that were produced in response to a number of environmental antigens other than that which was used for immunization. Numerous studies indicate increased susceptibility of vitamin A deficient animals to infection (Sherman and Burtis, 1927-28, Cohen and Elin, 1974; Krishnan et al., 1976; Tengerdy and Brown, 1977). Increased antigenic exposure of vitamin A deficient animals has been shown to be due, in part, to a reduced local resistance at the site of entry by infective agents (Darip et al., 1979). Vitamin A plays an essential role in maintenance of the integrity of anatomical barriers, such as epithelial and mucosal surfaces. If a vitamin A deficiency state develops, the specialized epithelium lining the respiratory and urinary tracts, eyes, and glands undergoes metaplastic conversion into keratinized stratified squamous epithelium (Wolf, 1984). DeLuca et al. (1980) have demonstrated increased perme-

ability of tracheal epithelium during vitamin A deficiency. Other workers

have observed secretory cells replaced by cells that lack the capacity to

produce mucous (Rojanapo et al., 1980) and lysozyme (Sullivan and

Manville, 1937). In addition, Sirisinha et al. (1980) found reduced

secretory IgA in the intestinal fluid of vitamin A deficient rats. In the

present study, I detected a small but significant decrease in intestinal

SIgA concentrations in vitamin A deficient chicks. The results of these

two studies indicate a reduced secretion of SIgA by epithelial cells

during vitamin A deficiency. Reduction in mucous, lysozyme and SIgA

secretion, as well as alterations in epithelium permeability, would allow

microorganisms to penetrate mucosal surfaces more easily. Other macro-

molecules, such as dietary proteins, pollen, etc., which are normally

excluded by the mucosal barrier also may cross the mucous membrane.

Continual bombardment of impaired mucosal tissue in vitamin A deficient

chicks would result in stimulation of systemic lymphoid tissue and result

in hypergammaglobulinemia.

Vitamin A deficiency may reduce the synthesis and concentrations of

immunoglobulins. This impairment may not be detected, however, due to

antigenic challenge of infection which occurs during vitamin A deficiency.

In a study by Chandra (1972), malnourished children with concurrent or

recent history of infection had tremendously increased serum immuno-

globulin concentrations compared with uninfected malnourished children.

Those children who were also malnourished but exhibited no evidence of

infection had significantly decreased serum IgG and IgA concentrations

when compared to healthy uninfected control children. In the present

study, the increased immunoglobulin concentrations observed in vitamin A deficient chicks may have been in response to more antigenic exposure of these birds as compared with vitamin A adequate chicks. The use of germ-free or gnotobiotic chicks would make it possible to separate the effect of vitamin A deficiency and environmental antigens on the immune system. Vitamin A deficiency may actually impair immunoglobulin production, but given the massive and prolonged exposure to antigens in these animals, accelerated production could occur despite the suppressing effect of vitamin A deficiency.

Infection per se can act as an immunosuppressant, irrespective of nutritional status. Infectious illnesses have been shown to deplete nutrient stores (Chandra and Newberne, 1977), alter the proportions of lymphocyte subpopulations (Coovadia et al., 1974), and impair the ability of lymphocytes to respond in vitro to mitogenic stimulation (Kantor, 1975). These alterations also have been reported to occur as a result of vitamin A deficiency. If an infection is neither recognized nor considered when the effect of nutritional status on immune function is being evaluated, infection-induced changes in immune function may erroneously be ascribed to the vitamin A deficiency. Imbalances in immune function due to subclinical infections and secondary nutritional disorders could help explain some of the discrepancies among host responses to humoral and cell-mediated functions during vitamin A deficiency that have been reported.

IgG is the vehicle of passive humoral immunity in the neonatal chick (Higgins and Calnek, 1975b). At hatch, the concentration of serum IgG is

high. The concentration increases for a few days after hatch due to the continued passage of maternal antibodies from the yolk sac to the serum. The yolk sac is fully absorbed within a few days after hatching. The concentration of passively acquired antibody in the serum of day-old chicks is approximately the same as in the serum of the dam (Allan et al., 1978). After the initial increase, the IgG concentration decreases during the first 2 weeks of life. From 2 to 4 weeks of age, the IgG concentration remains relatively constant. After 3 or 4 weeks of age, the IgG concentration increases as the chick matures (Higgins and Calnek, 1975b). In the present study, serum IgG concentrations increased steadily in chicks from 2 to 7 weeks of age. This increase was probably in response to the primary vaccination with NDV concurrent with immunological maturity.

IgM and IgA are absent or found in low concentrations in serum of the day-old chick (Leslie et al., 1976). Each appear, however, before the second week of age. Concentrations of IgM and IgA steadily increase as the chick matures (Higgins and Calnek, 1975b; Gillette, 1980). Serum IgM and IgA concentrations in the present study increased progressively from 2 to 6 weeks of age. At 7 weeks of age, a decline in serum IgM and IgA concentrations from the previous week was observed. The decline occurred one week after a sharp increase in IgG concentration was observed. In a similar study reported by Higgins and Calnek (1975a), serum IgM and IgA concentrations increased in 5-week-old chicks inoculated with Marek's disease virus. After IgM and IgA concentrations peaked 12 days after inoculation, serum IgM and IgA concentrations declined. One week after

these immunoglobulin concentrations began to fall, IgG concentrations increased rapidly. The decline in IgM and IgA concentration may reflect class switching from IgM and IgA to IgG production.

Quantitation of immunoglobulins in serum of NDV inoculated chicks from 2 to 7 weeks of age, gave results that exceeded those reported by other workers for chickens of the same age (Higgins and Calnek, 1975b; Leslie et al., 1976; Chhabra and Goel, 1980; Gillette, 1980). Low immunoglobulin concentrations were reported by researchers who used birds obtained from specific pathogen-free flocks, unvaccinated, and/or reared in conditions free from exposure to avian pathogens. Conventionally raised or viral inoculated birds exhibited high immunoglobulin concentrations (Higgins and Calnek, 1975a; Rees and Nordskog, 1981; Prescott et al., 1982). Values reported in the latter studies more closely resembled the concentrations observed in the present study. Chicks in the present study were vaccinated and, in addition, their unconventional diet may have predisposed them to greater antigenic stimulation. Both of these factors may have contributed to higher than normal serum immunoglobulin concentrations. Variation in immunoglobulin concentrations may also be caused by differences in the method used for quantifying immunoglobulins. Many of the proteins used to quantitate chicken immunoglobulins are not commercially available. Therefore, most laboratories isolate and characterize their own chicken proteins and produce their own monospecific antisera. This may result in variations among laboratories and the subsequent values reported.

In the research reported here, concentrations of all three immuno-globulins increased dramatically from prevaccination concentrations by 8 days after a second vaccination with NDV. IgG and IgM concentrations as high as those observed in the present study (18.8 and 8.74, respectively) were also reported by Higgins and Calnek (1975a) for 6-week-old chickens inoculated with Marek's disease virus. Serum IgA concentrations in their study also increased from preinoculation concentrations but values were much lower (.22 mg/ml) than those observed in the present study (3.33 mg/ml).

Evidence has accumulated that supports the existence of a local immune system in chickens distinct from that responsible for the formation of circulating antibody. Research has shown that the ability of chickens to be protected from reinfection with NDV is more highly correlated with prior exposure of the respiratory epithelium to the infectious agent than with concentration of serum antibody (Levy et al., 1975). Antibodies against a number of bacteria and viruses have been identified in external secretions (Ewert et al., 1977; Leslie et al., 1976). Immunoglobulins with antibody activity against infectious agents probably play an important part in the resistance of mucous membranes to colonization and absorption of potentially dangerous antigens.

Profound changes in the epithelium or mucous membranes are a characteristic feature of vitamin A deficiency. Presumably, alterations in the integrity of the epithelium interferes with the ability of the vitamin A deficient animal to resist infection. A reduction in the synthesis and/or

secretion of immunoglobulins by epithelial cells may contribute to impaired local immunity in vitamin A deficient chicks.

Determining the concentration of immunoglobulins in secretions is difficult because of the possible contamination of the sample with serum. Also, it is difficult to obtain enough fluid to accurately measure immunoglobulins, which usually are present at low concentrations in tissues. In addition, a reliable reference standard is required. The technique of irrigating the lumen of the respiratory and intestinal tracts with saline and then concentrating the recovered fluid has been used in studies to determine immunoglobulin concentrations of these tissues. Concentrations of immunoglobulins, then, are expressed as a percentage of total protein (Sirisinha et al., 1980) or relative to an undisclosed standard (Chhabra and Goel, 1980). Techniques that rely on standard-ization of immunoglobulin with respect to protein content of fluid recovered may give an erroneous representation of the immunoglobulin concentrations in some secretions.

In order to accurately compare immunoglobulin concentrations among chicks in the present study, I took approximately the same portion of intestine and trachea from each bird and expressed the immunoglobulin concentration as a percentage of total protein/g of tissue. To my knowledge, immunoglobulin values of various secretions have not been reported in this manner previously. Therefore, immunoglobulin values in secretions in the present study cannot be compared directly with published values. In general, however, the relative concentrations of the immuno-globulin classes in secretions agree with those in other studies. For

example, Chhabra and Goel (1980) detected an IgA to IgG ratio of .22 in tracheal washes. In the present study, the average IgA to IgG ratio in trachea was .28. All three immunoglobulin classes were detected in intestine and trachea.

In general, a decreased SIgA concentration, with respect to other proteins in the trachea and intestine of vitamin A deficient chicks, was observed after secondary vaccination with NDV in the present study. Because the concentration of total protein was normal or increased in intestine and trachea of vitamin A deficient chicks as compared with controls, the low concentration of SIgA observed was probably, in part, selective and not entirely due to a general reduction of all protein in these tissues. The reduction of SIgA may have been the result of selective depression of IgA synthesis in submucosa or of secretory component production by epithelial cells (Sirisinha et al., 1980). Depressed IgA synthesis may be due, in part, to a defect in the ability of lymphocytes, committed to IgA synthesis, to localize in submucosa (McDermott et al., 1982).

Impaired immunoglobulin production may contribute to an increased frequency of infection often associated with vitamin A deficiency. The small amount of luminal SIgA observed may fail to prevent mucosal binding of bacteria and enterotoxin. Systemic spread may also occur more easily because of the reduced efficiency of the mucosal barriers in checking pathogenic organisms from penetrating respiratory and gastrointestinal epithelium.

In contrast to the low SIgA concentrations, significantly increased concentrations of IgM and IgG were found in intestine and trachea of vitamin A deficient chicks after secondary vaccination with NDV compared with controls. The origin of these immunoglobulins would be important, however, in determining vitamin A's role in host immune defense. Since portions of the whole tissue were examined, the immunoglobulins detected could have been synthesized locally or systemically. Indeed, the concentrations of IgG, IgM, and SIgA in secretions generally paralleled the immunoglobulin concentrations in serum. The increased IgM and IgG may be attributed to the antigenic challenge provided by continual bombardment of epithelium with potentially pathogenic organisms. However, the reduction in SIgA appears to be selective since the concentrations of other immuno-globulin classes in tissues from vitamin A deficient chicks were increased as compared with controls.

To my knowledge, the effect of a vitamin A deficiency on the concentration of SIgA in bile has not been examined previously. Five weeks after a primary vaccination with NDV, a partial vitamin A deficiency did not significantly affect the concentration of SIgA in bile. Eight days after a second local and systemic vaccination with NDV, biliary SIgA concentrations were considerably greater in vitamin A control chicks than were prevaccination concentrations in the same treatment group. However, a small increase or decline in biliary SIgA concentrations was observed in vitamin A deficient chicks after the second vaccination. The more severe the deficiency the greater the decrease in the SIgA concentration in bile.

Total biliary protein increased in all chicks after secondary vaccination with NDV, regardless of dietary treatment. Because the total biliary protein concentration of vitamin A deficient chicks was normal or only slightly lower than that of controls, the marked reduction in SIgA observed in bile of vitamin A deficient chicks could not be explained on the basis of a general impairment of protein synthesis. Similarly, the differences in SIgA concentrations could not be explained by a dilution or concentrating effect by the gall bladder. The gall bladder of the chicken has the capacity to concentrate hepatic bile 6 to 10 times (Schmidt and Ivy, 1937). In the current research, only a 2 to 3% decrease in dry matter content of bile was observed in vitamin A deficient chicks as compared to the dry matter content of bile obtained from chicks fed adequate vitamin A diets.

Although these results clearly indicate decreased biliary SIgA in vitamin A deficient chicks after secondary vaccination with NDV, the origin of this immunoglobulin is important in determining the function of vitamin A in secretory immunity. The bulk of evidence suggests that the immediate source of biliary SIgA is polymeric IgA in the circulation, which is actively and selectively transported from blood to bile by the liver (Rose et al., 1981). The mechanism of transport of IgA seems to involve secretory component (Hall, 1981; Peppard et al., 1983). If the mechanism of transport of polymeric IgA from serum were impaired, IgA would accumulate in the serum (Rose et al., 1981). A significant increase in serum IgA concentration did occur in retinol deficient chicks when compared with retinol controls. However, in most cases a normal or low

serum IgA concentration was observed in RA deficient chicks when compared with RA controls.

IgA in the circulation appears to be derived from IgA-producing plasma cells in the lamina propria of the intestinal tract (Manning et al., 1984). The IgA-producing plasma cells arise from precursors in the gut-associated lymphoid tissue in response to antigens in the lumen of the gut (Weisz-Carrington et al., 1979). The precursors are lymphocytes committed to IgA synthesis, which travel from the gut-associated lymphoid tissue to the blood and eventually localize in mucosal lamina propria. Here, they mature to plasma cells and secrete IgA antibody. Any defect in homing of these lymphocytes would lead to impaired SIgA production and predispose the host to a variety of infections. McDermott et al. (1982) examined the intestinal localization of mesenteric lymph node lymphoblasts in vitamin A deficient rats. In this study, lymphoblasts isolated from intestinal lymph nodes of vitamin A deficient and control rats were labeled with 125-I deoxyuridine and injected into recipient animals. On the basis of 125-I activity, a smaller proportion of lymphoblasts obtained from vitamin A deficient rats localized within the gut of recipient rats as compared with cells derived from donor vitamin A control rats.

Factors determining the localization of normal lymphoblasts in tissues are poorly understood. Selective localization of lymphocytes in the intestine may involve interaction of lymphocyte cell surface glyco-protein receptors with recognition sites on the intestinal cell endo-thelium (Chin et al., 1980). There are reports of cell membrane changes

during vitamin A deficiency which occur as a result of a disruption of glycoprotein synthesis (DeLuca, 1977).

An alternative hypothesis supporting the impaired localization of lymphocyte cells derived from vitamin A deficient animals may be defective interactions among mucosal lymphocyte subpopulations. The role of T helper cells in the localization of IgA precursor cells in mucosal tissues remains to be determined. Whether T cells influence B lymphocyte locali- zation was studied in animals treated with anti-Thy 1 and complement (Bienenstock et al., 1983). Selective localization of B lymphoblasts in the intestine occurred in the absence of T cells. This indicates that T cells were not necessary for mucosal B lymphoblast localization.

Organized mucosal lymphoid tissue, such as the Peyer's patches, is enriched in the early progenitors of IgA plasma cells. These cells eventually appear in the gut as IgA plasma cells. Research with Peyer's patches has resulted in the discovery of a T cell subset which converts immunoglobulin M synthesizing B cells to IgA synthesizing B cells (Elson et al., 1979). A defect in the function or number of this Peyer's patches T cell subset, which controls the development of IgA plasma cells, might cause a decrease in the number of gut associated lymphoid cells which become committed to IgA synthesis and, therefore, intestinal localization. However, once these converted cells migrate from the Peyer's patches to the lamina propria other specialized T cells may participate in their proliferation and maturation to IgA secreting plasma cells.

A major proportion of lymphocytes in mucosal lamina propria are T cells (Arnaud-Battandier et al., 1980). The functions of lamina propria T

cells are not well-understood. Impaired SIgA production and inadequate mucosal immunity in vitamin A deficiency may be due to a numerical or functional inadequacy of T cells which have a helper or a suppressor function for B cells.

Indeed, a loss of T cell function has been associated with vitamin A deficiency. A vitamin A deficiency causes a loss of cellularity in thymus and bursal tissue (Bang et al., 1972; Bang et al., 1973). Studies of children showing signs of vitamin A deficiency showed that T lymphocyte concentrations in peripheral blood were lower than those of controls (Bhaskaram and Reddy, 1975; Kutty et al., 1981). Recently, Malkovsky et al. (1983) revealed that vitamin A acetate-fed mice had a significantly higher proportion of Lyt 1.1 positive cells in their lymph nodes than nonsupplemented control mice. The action of vitamin A acetate in enhancing immunologic reactivity may be mediated by its ability to increase the proportion of helper T cells rather than suppressor or effector cell types. The rate of T lymphocyte transformation in response to mitogenic stimulation has been shown to be impaired during vitamin A deficiency (Nauss et al., 1979).

Sources of biliary SIgA other than gut associated lymphoid tissue have been proposed. Synthesis of immunoglobulins by the chicken gall bladder itself has been suggested (Leslie et al., 1976). Lymphoid cells that produce IgA have been detected in the gall bladder of adult chickens (Leslie et al., 1976). Any defect in the number and function of these cells as a consequence of vitamin A deficiency would also contribute to reduced biliary SIgA.

Supplementing and subsequently withdrawing RA from the diets of retinol-deficient chicks was used in the present study to induce rapid vitamin A deficiencies. Retinoic acid is a metabolite in the activation and elimination pathways for retinol and is normally not consumed in the diet. It is important, therefore, to determine if the activity of RA and retinol, in host defense, are equivalent.

Retinoids have been used in experimental animal systems to determine their potential as anti-carcinogenic agents. Retinoic acid as well as retinol has been reported to inhibit the growth of transplantable tumors (Trown et al., 1976) and to augment tumor immunity following administration of viruses (Glaser and Lotan, 1979). Yet, few studies have been reported that compare the activity of retinol and RA in humoral and cell-mediated immune functions.

Sirisinha et al. (1980) noted that SIgA concentrations in intestinal fluid of vitamin A deficient rats increased rapidly after they consumed a diet supplemented with retinyl palmitate. When these rats were fed diets supplemented with RA, intestinal SIgA concentrations never increased to the level observed after they were fed the retinyl palmitate supplemented diets. Reduction in production of SIgA may predispose animals to infection, thereby resulting in increased serum immunoglobulin concentrations. However, the results reported by Sirisinha et al. (1980) indicated that serum IgG and IgA concentrations were not changed significantly by a change in the source of vitamin A added to the diet.

In the present study, chicks depleted of hepatic retinol reserves were fed diets supplemented with 2 ug of vitamin A/g of diet either in the

form of retinol or RA. No significant difference in intestinal and tracheal SIgA concentrations was observed between chicks fed 2 ug RA/g of diet and chicks fed the same amount of vitamin A in the form of retinol. However, SIgA concentrations were significantly greater in bile of chicks fed diets supplemented with RA as compared with chicks fed diets supplemented with retinol. These results suggest that the secretory immune response was not impaired by supplementing diets with vitamin A in the form of RA. In addition to the enhanced SIgA concentration, serum IgG, IgM, and IgA concentrations were also significantly greater in chicks fed 2 ug of RA/g of diet as compared with chicks fed 2 ug of retinol/g of diet.

Increased immunoglobulin concentrations may indicate increased antigenic exposure of chicks fed RA as the vitamin A source. Increased antigenic exposure can result from alterations in epithelial structures, which provide a portal of entry for infectious agents. However, this explanation is not consistent with the observation that RA is the form of vitamin A responsible for supporting epithelial differentiation. It has been demonstrated that administration of RA to vitamin A deficient animals reverses most epithelial lesions brought about by the deficiency (Rojanapo et al., 1980). In addition, RA has been shown to be 10 times more active than retinol in suppressing keratinization of tracheas in organ culture (Sporn et al., 1975). Therefore, it seems that increased immunoglobulin concentrations cannot be explained solely on the basis that RA was unable to maintain local anatomical barriers.

A contributing factor for the possible inability of dietary RA to maintain anatomical barriers as well as retinol is that, RA, unlike retinol, is not stored in the body and is rapidly metabolized (Krishnamurthy et al., 1963). The dietary requirement for RA may have been increased in chicks in the present study. In order to deplete hepatic vitamin A reserves, chicks were fed a vitamin A-free diet for the first 2 weeks of age. During this deficiency period, chicks were susceptible to infectious agents. Infectious disease is associated with rapid utilization of body stores of nutrients (Beisel, 1980). Germfree experiments have shown that growth can be maintained on extremely low concentrations of retinol if the animal is kept in a sterile environment. Supplementation of the diet with retinol at a concentration that is just adequate for growth in the germfree animal will not support growth in conventionally raised animals (Rogers et al., 1971). In the present study, 2 ug of RA/g of diet fed to retinol deficient chicks may have been metabolized and excreted so rapidly that only a limited amount of the material was able to fulfill vitamin A's function in maintenance of epithelial tissue. A slight but significant decrease in the weight of the intestine obtained from chicks fed 2 ug of RA/g of diet compared with chicks fed the same amount of vitamin A as retinol is consistent with this notion.

The serum antibody response to secondary vaccination with NDV was greater in chicks fed RA control diets than in chicks fed retinol control diets. Greater serum immunoglobulin and biliary SIgA concentrations concurrent with greater serum antibody response suggest that RA may be

superior to retinol in systemic as well as local immune function. Further studies using germfree or gnotobiotic animals are needed to determine whether increased immunoglobulin concentrations in RA-fed animals, as compared with retinol-fed animals, is a direct effect of RA on the synthesis and secretion of immunoglobulins or an indirect effect due to increased antigenic stimulation.

The results of this research reveal that chicks suffering from vitamin A deficiency show profound disturbances in immunoglobulin metabolism. Although the concentrations of IgG and IgM in serum and secretions of vitamin A deficient chicks were increased, the blunted antibody response to NDV demonstrates that these increases do not always signify a completely intact humoral immune system. The complex factor of high frequency of intercurrent infection in vitamin A deficient animals and how this influences immunoglobulin production needs to be clarified by further research.

## SUMMARY

The effect of vitamin A deficiency on the local and systemic immune system of broiler chicks was examined. Day-old chicks were fed a vitamin A-free diet to deplete them of hepatic vitamin A reserves. At 13 days of age, the vitamin A deficient chicks were fed diets supplemented with either 2 ug retinol, 2 ug all-trans retinoic acid (RA), or 0.2 ug retinol/g of diet. Chicks were vaccinated intraocularly and intranasally with a live B1 strain of Newcastle disease virus (NDV) at 2 weeks of age. Serum samples were obtained weekly for determination of serum IgG, IgM, and IgA concentrations. Immunoglobulins IgG and IgM were determined by radial immunodiffusion using monospecific antisera to IgG and IgM, respectively. IgA concentrations were determined by rocket immunoelectrophoresis using monospecific antisera to IgA.

During a 5 week period after chicks were given a primary vaccination with NDV, serum IgG, IgM, and IgA concentrations increased in retinol deficient chicks and in chicks fed RA control diets, as compared with those observed in chicks fed 2 ug retinol/g of diet. At 7 weeks of age, all three classes of immunoglobulin were significantly greater in the retinol deficient (0.2 ug/g of diet) and RA control chicks (2 ug/g of diet) as compared with retinol control chicks (2 ug/g of diet). There were no significant dietary treatment effects on the concentration of serum antibody, in response to the primary vaccination with NDV, as determined by hemagglutination inhibition assay.

At 7 weeks of age, RA-fed chicks were further divided into three treatment groups. One group remained on the RA control diet (2 ug/g of

diet); the other two groups were fed either 0 or 0.2 ug RA/g of diet.
Chicks fed 0.2 ug of retinol/g of diet were changed to a vitamin A-free
diet. Chicks fed the retinol control diet (2 ug retinol/g of diet)
remained on this diet throughout the experiment. At 2, 4, or 6 days after
final diet changes were made, chicks were revaccinated with an attenuated
La Sota strain of NDV by intraocular, intranasal, and intramuscular
routes. In general, prolonging the vitamin A-deprivation period 2 or 4
additional days before vaccination did not affect the pattern of responses
to diet treatments. In most instances, 2 days of vitamin A-deprivation
before the second dose of NDV was given was a sufficient period of time
for the development of differences among dietary treatment groups.

Eight days after the second NDV vaccination, serum IgG and IgM
concentrations were significantly higher in chicks fed a retinol or RA
deficient diet as compared with chicks fed either 2 ug of retinol or RA/g
of diet, respectively. The response of serum IgA to secondary vaccination
in vitamin A deficient chicks was not consistent. Serum IgA concentra-
tions were greater in retinol deficient chicks after revaccination, but
lower or similar amounts of IgA were detected in the serum of RA deficient
chicks as compared with RA controls. Despite an increase in serum
immunoglobulin concentrations, serum antibody titer to the second vaccina-
tion with NDV was significantly decreased in chicks fed a vitamin A
deficient diet as compared with controls.

Secretory IgA (SIgA) concentration in bile obtained from gall
bladders of vitamin A deficient chicks 8 days after the second vaccina-
tion, were significantly lower than that of controls. A decreased SIgA

concentration was also observed in intestine and trachea after secondary vaccination with NDV in vitamin A deficient chicks as compared with vitamin A controls. In contrast to the low SIgA concentrations in tissues, significantly increased concentrations of IgM and IgG were found in intestine and trachea of vitamin A deficient chicks as compared with controls.

Serum IgG, IgM, IgA, and biliary SIgA concentrations before and after secondary vaccination with NDV were significantly greater in chicks fed diets supplemented with 2 ug RA/g of diet as compared with chicks fed an equivalent amount of vitamin A in the form of retinol. Serum antibody response to the second NDV vaccination was significantly greater in chicks fed 2 ug of RA/g of diet as compared with chicks fed 2 ug retinol/g of diet. These results suggest that RA may be superior to retinol in support of systemic and local immune functions.

A reduction in SIgA concentration in tissues and a selective decrease or no change in serum IgA concentration, after secondary vaccination with NDV, may indicate impaired IgA production. The reduction in SIgA observed may contribute to an inability of mucosa to prevent colonization and binding of potentially pathogenic organisms. In addition, vitamin A deficiency is associated with a reduced efficiency of mucosal barriers to prevent penetration of infective agents (Darip et al., 1979). Reduction in SIgA as well as alterations in epithelium permeability would increase antigenic exposure of vitamin A deficient chicks to infective agents. Increased antigenic exposure of lymphoid tissue would result in hypergammaglobulinemia. Vitamin A deficiency may actually impair

immunoglobulin production but, given the massive and prolonged exposure to antigens, accelerated production may also occur. Increased immunoglobulin concentrations in the presence of reduced antibody production observed in vitamin A deficient chicks is consistent with this concept.

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## ACKNOWLEDGMENTS

## APPENDIX A:  THE HEMAGGLUTINATION INHIBITION TEST

### Preparation of the Working Hemagglutinin

Ulster strain of Newcastle disease virus, harvested in allantoic fluid from embryonating eggs, was inactivated by the addition of .1% formalin. The fluid was diluted 1 to 40 with saline/cox buffer, pH 7 (9.2 g $NaH_2PO_4$ and 34.6 g $Na_2HPO_4$ in 2 liters $H_2O$), to give a hemagglutination (HA) titer of 20 units.

### Preparation of Red Blood Cells

Fresh chicken blood was drawn into a heparin-containing syringe. An equal amount of phosphate-buffered saline (PBS, pH 7) was added to the blood and the erythrocytes (RBC) were separated from other blood components by centrifugation. The pooled RBCs were washed twice in PBS and once in alsevers solution (20.5 g dextrose, 8 g sodium citrate, 4.2 g sodium chloride in 1 liter of $H_2O$). The washed packed RBCs were dispersed as a 10% suspension in alsevers solution.

### Hemagglutination Inhibition Test Methods

The test was carried out on round-bottom linbro microtiter test plates (Flow Laboratories, Hamden, CN). The first well of the microtiter test plate received .08 ml of saline/cox buffer; all other wells contained .05 ml of saline/cox buffer. In the second well, .05 ml diluted virus (1 to 40) (20 HA units) were added. A second sample of virus was made by further diluting the 1 to 40 antigen 1 to 2 with saline/cox buffer. An aliquot (.05 ml) of this 1 to 80 (10 HA units) virus suspension was added to the remaining six wells of each test row.

Undiluted serum samples (0.2 ml) obtained from 2 to 7 weeks of age chickens were added to the first row of wells, which allowed these samples to be titrated 1 to 5 to 1 to 640. Serum samples obtained from chicks that received a second vaccination of NDV were diluted 1 to 40 in PBS, pH 7.2, before being added to the first well in aliquots of 0.1 ml. This allowed these samples to be titrated 1 to 40 to 1 to 5120. The serum was serially diluted with an automatic dilution unit (Cooke Laboratory Products, Alexandria, VA). After the serum was diluted in the wells of the plate, one drop of a 10% suspension of RBC was added to each well. The plates were incubated at room temperature (24 C) for 1 hour.

A positive inhibition reaction occurred when a free-flowing button of cells formed in the bottom of the well. Results were expressed as geometric mean titer (GMT), which represented the reciprocal of the greatest dilution of serum showing inhibition of agglutination with antigen.

APPENDIX B:  PREPARATION OF TRACHEA AND
INTESTINAL TISSUES

Immediately after the chicks were killed, 20 cm of trachea and
intestine were removed and each individual tissue was placed in preweighed
50 ml screwcap plastic tubes containing 10 ml cold (4 C) phosphate-
buffered saline (PBS, pH 7.2). The PBS contained .01% $NaN_3$ as a preserva-
tive and $10^{-3}$ m phenylmethanesulfonyl fluoride (Sigma, St. Louis, MO) as a
trypsin inhibitor. The tissue samples that were contaminated with blood
were rinsed with cold PBS before placing them in plastic vials. Tissue
weights were determined by weighing the preweighed plastic tubes after
tissues were enclosed.

Individual tissues were homogenized with a Brinkman Polytron
Homogenizer in the presence of 10 ml of PBS in the same plastic tubes used
for tissue collection.

The homogenate was centrifuged at 4 C (47,800 x g) for 30 minutes.
The supernatant was removed and the total volume measured.

Individual tissue supernatants were stored at -20 C until analyzed.
Aliquots of these supernatants were used for the determination of IgG, IgM
and IgA in intestine samples and IgG and IgM for trachea samples. Tissue
supernatants which contained excessive blood or bile were removed from the
analysis.

For IgA determination, 7 ml of supernatant from each trachea sample
homogenized were frozen and lyophilized. The lyophilized samples were
resuspended in 1 ml of distilled $H_2O$ and samples within each pen were
pooled. This resulted in three to four samples per pen assayed by rocket

immunoelectrophoresis. The high salt concentration produced by concentrating samples caused excessive heating of the agarose gel during electrophoresis, which resulted in water condensing on the surface of the gel and caused the precipitin peaks to be poorly defined. In order to remove the excess salt, samples were dialyzed against barbital-acetate buffer, pH 9, for 72 hours. The concentrated, pooled and dialyzed preparations of supernatant from homogenized trachea samples were used for determination of trachea IgA concentrations.

APPENDIX C:   QUANTITATION OF CHICKEN IgM AND IgG

Radial Immunodiffusion

Chicken IgG and IgM concentrations in serum and tissues were esti-
mated by radial immunodiffusion.

Antiserum

Goat antiserum monospecific for chicken IgG was obtained from Pel-
Frez (Rogers, AR).  Goat antiserum monospecific for chicken IgM was
obtained from Miles Laboratories (Elkhart, IN).  Each antiserum was tested
at several dilutions in order to determine the optimal antibody concentra-
tion to incorporate into agarose gels for use in each test.  A desirable
antiserum concentration was one which used the least amount of specific
antiserum and still gave ring precipitates which were easily readable in
the range of protein concentrations to be tested.

IgM and IgG antisera incorporated into 1% agarose at .5% proved best
for all trachea and intestine determinations.  The concentration of
antiserum required for serum determinations, however, increased with age
of the chicks.  Determination of IgG and IgM in serum samples from chicks
2 to 4 weeks of age required .5% IgG and IgM antisera to be incorporated
into agarose gels.  Agarose gels for determination of serum IgG and IgM
obtained at 5 and 6 weeks of age required 1% antiserum.  The optimum
antiserum concentration in 1% agarose gels for serum obtained 0, 2, 4, and
6 days after final diet changes were made was 1.5% for IgG and 2% for IgM
determinations.

## Preparation of agarose gels

Antibody-containing agarose diffusion plates were prepared by adding 1 g of agarose (Seakem LE, Marine Colloids, Rockland, ME) to 100 ml of phosphate-buffer, pH 7.2 (containing .05 m potassium phosphate, .15 m sodium chloride and, as a preservative, 0.5 g sodium azide). The agarose beads were allowed to swell in the buffer for 30 minutes after which time the suspension was heated until all the agarose was dissolved. Distilled water was added to replace losses due to evaporation. When the agarose was completely in solution, agarose solution was transferred to a 57 C water bath. When the solution reached 57 C, appropriate volumes of monospecific antisera were added to the agarose solution. Twenty-one milliliters of the antibody-containing agarose were delivered with a preheated pipette onto Gelbond film agarose gel support medium (FMC Corporation, Marine Colloids, Rockland, ME), measuring 100x150 mm and 0.2 mm thick. The antiserum agarose mixture was allowed to harden on a leveled table at room temperature. Prepared gels were kept in a moist chamber at 4 C until used.

## Application of test samples to agarose gels

Six rows of seven circular wells were cut in the agarose layer using a 4 mm or 2.5 mm well cutter (LKB, Gaithersburg, MD).

IgM concentrations were determined by filling 4 mm wells with 10 ul serum samples. Serum in 5 ul aliquots was applied to 2.5 mm wells for IgG determinations. IgG concentrations in trachea and intestine were determined by filling 4 mm wells with 10 ul aliquots of test samples. IgM concentrations in trachea and intestine samples were determined by filling

4 mm wells with 10 ul aliquots and then allowing the sample to be absorbed by the gel. The sample application was then repeated so that each well received a total of 20 ul of test sample. All samples were tested in duplicate on different agarose gel plates on the same day.

## Preparation of test samples

Serum samples obtained weekly from 2 to 6 weeks of age were tested undiluted. Serum obtained at 7 weeks of age was diluted 1 to 4 in PBS pH 7.2. Serum obtained 2, 4, and 6 days after final diet changes occurred were diluted 1 to 8 in PBS before assay.

## Reference standards

Chicken IgG and IgM were prepared from chicken serum using methods described in Appendix D. Four wells on each plate were filled with serial twofold dilutions of immunoglobulin standards or reference serums. Standards containing an initial concentration of 5 and 10 mg IgG/ml were used in tests for determining IgG in serum obtained from 2 to 5 weeks and after 5 weeks of age, respectively. A reference serum containing 3 mg IgM/ml was used in four serial dilutions for determination of IgM in all serum samples tested. Initial standards for determining immunoglobulin concentrations in intestine and trachea were 2.5 mg IgG/ml and .75 mg IgM/ml.

The plates were incubated in moist chambers for 24 and 72 hours at 37 C for IgG and IgM determinations, respectively.

## Drying and staining of agarose gel plates

After incubation, the unprecipitated proteins were leached from the
gel by washing for 24 hours in PBS, pH 7.2. The gels were then soaked for
30 minutes in distilled water in order to remove the salt. The wet gels
were overlaid with moistened Whatman #1 filter paper followed by four dry
sheets of Whatman #1 filter paper. An even pressure was applied to the
gel by placing 500 g weight on top of the absorbent material. After 15
minutes, the weight and absorbant layers were removed from the surface of
the gels. The thin, collapsed agarose gel layer was then dried at room
temperature.

Precipitation in dried gels was stained with Crowles Double stain
(containing 250 mg Crocein Scarlet (Eastman Kodak Co.) and 15 mg Brilliant
Blue R (Sigman, St. Louis, MO) in 100 ml of 5% acetic acid and 3%
trichloroacetic acid solution) for 30 minutes. Excess stain was removed
with a 0.3% acetic acid solution before final drying.

## Standard curves

The diameters of the precipitation rings were measured with a vernier
caliper to the nearest 0.1 mm. The precipitin ring diameters were plotted
against the log concentrations of the standard immunoglobulin values of
the four, twofold dilutions and a standard line was drawn. The immuno-
globulin concentration of serum or tissues was determined by referring to
the standard curve.

APPENDIX D:  QUANTITATION OF CHICKEN IgA

Chicken IgA concentrations in serum, bile and tissues were determined by rocket immunoelectrophoresis.  Goat antisera monospecific for chicken IgA was obtained from Miles Laboratories (Elkhart, IN) and incorporated into 1% agarose at .5%.

The 1% antibody-containing agarose plates were prepared by dissolving agarose in barbital-acetate buffer, pH 9 (containing .02616 m sodium barbital (Mallinckrodt Inc., Paris, KT), .0216 m sodium acetate, and .01% $NaN_3$) and adding .5% monospecific antisera to chick IgA as described previously for radial immunodiffusion gels (see Appendix C for procedure). Twelve milliliters of agarose-containing antibody were pipetted onto support medium measuring 85x100 mm and 0.2 mm thick.

Two rows of 12 wells each (2.5 mm diameter, 5 mm between wells) were punched in the gel.  Five microliter samples were assayed.  Undiluted serum obtained from 2 to 6 weeks of age were tested.  Serum obtained at 7 weeks of age was diluted 1 to 4 in barbital acetate buffer, pH 9.  Serums obtained 2, 4, and 6 days after final diet changes were made were diluted 1 to 8.  Bile obtained from chicks at 7 weeks of age was diluted 1 to 30 and all other bile samples were diluted 1 to 60.  Intestine and trachea samples were prepared for IgA determination as described in Appendix B.

Four wells in each row on each plate were filled with 5 ul of serial twofold dilutions of reference bile standard.  The reference bile standard used was the partially purified, salted-out preparation of bile described in Appendix F.  This reference bile contains .93 mg IgA/ml as determined by rocket immunoelectrophoresis by using purified IgA as the standard (see

Appendix F for purification of IgA procedure). Four serial dilutions of bile reference containing .93 mg/ml were used for determination of bile and serum. The concentrations of IgA in intestine and trachea samples were determined by using the bile reference standard at an initial concentration of .233 mg IgA/ml.

Electrophoresis (LKB Multiphor System, LKB, Gaithersburg, MD) was started immediately after samples were added, at an applied voltage of 10 v/cm for 3 hours at 4 C. A maximum of three plates were run at one time with the sample wells at the cottodal end. The electrode vessels, containing 1 liter each of barbital-acetate buffer, pH -9, were joined to the agarose gels by means of five sheets of moistened Whatman #1 filter paper wicks.

After electrophoresis, the gel plates were washed, pressed, dried, stained, and destained as described previously for radial immunodiffusion gels (see Appendix C for procedure).

The distance from the top of the well to the precipitation peak was measured with a vernier caliper to the nearest .1 mm. Calibration curves were constructed by plotting log concentrations of bile standards versus precipitate distance. The linear standard curves were expressed in an equation of first degree and the concentration of IgA in the unknown samples were calculated from the height of the precipitate by solving the equation.

APPENDIX E:   IgG AND IgM ISOLATION AND PURIFICATION

## Salt Precipitation

Chicken serum was salted out with $Na_2SO_4$ in a manner similar to the method described by Benedict (1967). One hundred ml of chicken serum obtained from Gibco (Grand Island, NY) were centrifuged at 5000 x g for 30 minutes at 25 C. Three successive salt precipitations of serum were performed at room temperature by using final $Na_2SO_4$ concentrations of 18%, 14%, and 14%, respectively. The solid sodium sulphate was added slowly with stirring, to avoid localized higher concentrations of $Na_2SO_4$ and thereby prevent albumin precipitation together with the $\gamma$-globulins. After admixture, precipitation was allowed to proceed for 30 minutes. After each precipitation, samples were centrifuged at 5000 x g at 25 C for 30 minutes; the supernatants were discarded and the pellets resuspended in one-quarter the original supernatant volume in borate-buffered saline, pH 8.2 (containing 10.3 g $H_3BO_3$, 1.1 g NaOH, 7.85 g NaCl, and .01% $NaN_3$ per liter of $H_2O$). The resuspended final product was extensively (72 hours) dialyzed at 4 C against borate-buffered saline.

## Gel Filtration

A 1 ml sample (30 mg protein/ml) of the salt-precipitated, dialyzed globulin preparation was applied to a chromatography column (2x95 cm) (LKB 2137, LKB, Gaithersburg, MD) packed with S-300 Sephacryl (Pharmacia, Piscataway, NJ) and pre-equilibrated with borate-buffered saline, pH 8.2. Flow was maintained in a downward direction at a rate of 13.5 ml/hour and 2 ml fractions were collected. Elution of the protein was monitored by

absorption at 280 nm. Three protein peaks eluted from the column were pooled and concentrated by ultrafiltration in a 50 ml standard ultrafiltration cell (Model 52, Amicon Corporation, Danvers, MA) utilizing a PM 10 43 mm Diaflo ultrafiltration membrane. The concentrated protein fractions were sterilized by passage through a .45 and then a .2 μm disposable filter assembly (Acrodisc, Gelman Sciences, Ann Arbor, MI). Distribution of IgG, IgM, and IgA was evaluated by immunoelectroploresis (see Appendix H for procedure) against appropriate monospecific antisera. Immunoelectrophoretic analyses of the first peak with antiserum against whole chicken serum (Miles Laboratories, Elkhart, IN) revealed a single precipitin line. An identical immunoelectrophoretic picture was obtained with goat antichicken IgM. When, however, peak 1 was tested by rocket immunoelectrophoresis (see Appendix D for procedure) in 1% agarose containing .5% goat antichicken IgA and 1.5% goat antichicken IgG, small concentrations of IgA and IgG were detected, respectively. Similarly, peak 2 revealed a single precipitin line in immunoelectrophoresis against antiserum to whole chicken serum and an identical precipitin arc with goat antichicken IgG. However, considerable amounts of IgM were detected by rocket immunoelectrophoresis with 1% agarose gels containing 2% goat antichicken IgM, and a small amount of IgA was detected in agarose gels containing .5% goat antiserum to chicken IgA. Peak 3, when examined by immunoelectrophoresis, revealed at least four precipitin lines with antiserum to whole chicken serum and one line with anti-IgG. No IgA or IgM was detected by rocket immunoelectrophoresis.

## Purification by Affinity Chromatography

Peak 1 containing predominantly IgM was further purified by passage over immunoabsorbant columns labeled with goat antiserum to IgG and rabbit antiserum to IgA (see Appendix G for procedure). Peak 2 which contained predominantly IgG was further purified by passage over immunoabsorbent columns labeled with goat antiserum to IgM and rabbit antiserum to IgA. The proteins eluted with PBS, pH 7.5 were tested for their purity by immunoelectrophoresis and rocket immunoelectrophoresis. The IgM and IgG preparations disclosed a single precipitin line with antiserum to whole chicken serum and an identical precipitin arc with antiserum to IgM and IgG, respectively. No other immunoglobulin classes were detected in each fraction by rocket immunoelectrophoresis.

The final protein concentration of each immunoglobulin fraction was determined by Lowry et al. (1951). The IgM preparation contained .8 mg protein/ml and was used to determine the concentration of IgM in a reference serum standard by rocket immunoelectropheresis (see Appendix G for procedure). The IgG preparation contained a final protein concentration of 10 mg/ml and was subsequently used as a protein standard for radial immunodiffusion tests.

## APPENDIX F: ISOLATION AND PURIFICATION OF CHICKEN IgA

### Salt Precipitation

One hundred milliliters bile were collected from the gallbladders of freshly killed chickens at a local processing plant. The bile was centrifuged at 13,000 x g for 30 minutes at 4 C. Ammonium sulphate was added to the bile at room temperature to 25% saturation to precipitate mucinous and pigmented material. These were removed by centrifugation at 1500 x g at 4 C. The supernate was removed and brought to 45% saturation to precipitate globulins. The resulting precipitate was removed by centrifugation at 1500 x g at 4 C. This precipitate was washed with 50% ammonium sulphate, centrifuged at 1500 x g at 4 C and resuspended in Tris buffer (.015 m tris and .15 m NaCl, pH 7.8). The suspension was dialyzed against this buffer for 72 hours. The salted-out preparation of bile was filtered through a .45 and .2 μm disposable filter assembly (Acrodisc, Gelman, Ann Arbor, MI) and used to immunize a goat (see Appendix I for procedure). The goat antiserum for chicken bile globulin was later used in immunoelectrophoretic tests to determine the purity of chicken bile IgA preparations.

When tested by immunoelectrophoresis, the bile globulin preparation produced a single precipitin arc against anti-IgA (see Appendix H for procedure). This same precipitin arc and a rapidly migrating component were precipitated by goat antichicken serum (Miles Laboratories, Elkhart, IN). These two precipitin arcs plus a third precipitin band was observed when the bile globulin preparation was tested against goat chicken bile globulin antiserum. The bile globulin preparations contained a small

amount of IgG as determined by immunoelectrophoresis with goat antichicken IgG. No IgM was detected by this method or by rocket immunoelectrophoresis using appropriate monospecific antiserum.

## Gel Filtration

The salted-out preparation of bile was subjected to gel filtration on Sephacryl S-300 (Pharmacia, Piscataway, NJ) on a 2x95 cm column (LKB 2137, LKB, Gaithersburg, MD) pre-equilibrated with Tris-NaCl buffer, pH 7.8. Five milliliters of bile prep (12.5 mg protein/ml) were applied to the column with an upward flow-rate of 14 ml/hour. The eluting protein was monitored by UV adsorption at 280 nm. The ascending portion of the leading peak was pooled and concentrated by ultrafiltration in a 50 ml standard ultrafiltration cell (Model 52, Amicon Corporation, Danvers, MA) by utilizing a PM 10, 43 mm Diaflow ultrafiltration membrane (Amicon Corporation, Danvers, MA).

Immunoelectrophoretic analysis with appropriate monospecific antiserum indicated that the pooled fractions from the first peak contained IgA but no IgG. However, when tested with antichicken serum or bile globulin antiserum, both the fast migrating component and IgA were still present.

## Purification by Affinity Chromatography

Since the second protein in the bile IgA preparation did not react with antiserum to immunoglobulin heavy chains, this component was removed from the IgA preparation by passage through an immunoadsorbent column labeled with goat antiserum to chicken IgA (Miles Laboratories, Elkhart,

IN) (see Appendix G for procedure). The protein that eluted with PBS, pH 7.5 containing 2.8 m KSCN, gave one precipitin arc with anti-IgA and an identical single precipitin arc with antiserum to chicken bile globulins and whole chicken serum. The final IgA preparation was concentrated by ultrafiltration to .46 mg protein/ml. The IgA preparation was dialyzed in barbital-acetate buffer, pH 9. The concentration of IgA in the bile globulin reference standard was determined by means of rocket immuno-electrophoresis and the purified IgA as standard (see Appendix D for procedure).

## APPENDIX G: AFFINITY CHROMATOGRAPHY

Affinity chromatography was used for removing contaminating anti-
bodies and for eluting specific antibodies. Immunoadsorbent columns were
prepared with goat antichicken IgG (Pel-Frez, Rogers, AR), goat anti-
chicken IgM (Miles Laboratories, Elkhart, IN), and goat antichicken IgA
(Miles Laboratories, Elkhart, IN).

### Activation of Sepharose

Ten ml of sepharose (4B-200, Sigma, St. Louis, MO) were mixed with an
equal volume of water and stirred in a 100 ml beaker. One gram of
Cyanogen bromide (Eastman Kodak, Rochester, NY) was pulverized with a
mortar and pestel under a ventilation hood and added to the sepharose
slurry. The pH of the slurry was increased immediately to 11 by the
addition of .5 m NaOH. The pH of the slurry was maintained between 10.5
and 11 by normal titration with .5 m NaOH. Ice was added occasionally to
the slurry to maintain the temperature near 20 C. The reaction was
complete when base uptake ceased (pH remained stable at 11) and no solid
CNBr remained. At this time, the slurry was poured into a sintered-glass
funnel and the gel was rapidly filtered with suction and washed with 250
mls of cold bicarbonate buffer, pH 9 (containing 32 g $NaHCO_3$, 22 g $Na_2CO_3$,
and 118 g NaCl in 4 liters of $H_2O$).

The gel was filtered to a moist cake and the outlet of the funnel was
covered with parafilm. Ten milliliters of the protein to be coupled (5-10
mg/ml) was dialyzed extensively (72 hours) in bicarbonate buffer, pH 9,
and added to the funnel and mixed immediately with a glass rod. The

slurry was poured into an erlenmeyer flask and the suspension was gently shaken on a mechanical shaker overnight at 4 C.

The gel was poured back into a sintered-glass funnel and the uncoupled protein washed and filtered through the funnel with coupling buffer. The gel was resuspended in a fourfold volume of 1 m ethanolamine, pH 8, in order to block excess active groups. The suspension was shaken 2 hours at room temperature. The excess blocking reagent was washed with bicarbonate buffer, pH 9, and filtered through the sintered glass filter, followed by alternate washings with 100 ml each of the following solutions cooled to 4 C: 0.1 m acetate buffer, pH 4 (containing 4.9 g sodium acetate, 2.3 ml glacial acetic acid and 29 g NaCl in 500 ml $H_2O$) and 0.1 m borate buffer, pH 8.5 (containing 1.55 g boric acid, 2.38 g sodium borate, and 29 g NaCl in 500 ml $H_2O$). The alternate washings were repeated four times, ending with borate buffer. The final washing of the immuno-adsorbent was carried out with phosphate-buffered saline (PBS, pH 7.5) (containing .05 m $KH_2PO_4$ and .5 m NaCl and .01% $NaN_3$). The packed immunoadsorbent was poured into a 10 ml plastic syringe and stored at 4 C.

<div style="text-align:center">

Adsorption and Elution of Proteins From the
Immunoadsorbent

</div>

The entire elution procedure was carried out at 4 C. The immuno-adsorbent gel was washed by gravity flow with 2.8 M KSCN in PBS, pH 7.5 until the optical density of the effluent read less than .05 at 280 nm. The dissociation agent was removed from the gel by washing the gel with PBS, pH 7.5. The presence of $SCN^{-1}$ was determined by adding one drop of 1% $FeCl_2$ and one drop of effluent. If a dark color resulted, then $SCN^{-1}$

was still present in the effluent and washing with PBS was continued until the $FeCl_2$ spot test indicated the absence of $SCN^{-1}$. Alternate washing of the immunoabsorbent was repeated with 2.8 KSCN in PBS, pH 7.5 and PBS, pH 7.5.

The protein fractions to be adsorbed were dialyzed extensively (72 hours) against PBS, pH 7.5 and added slowly to the immunoadsorbent column. The column was washed with PBS, pH 7.5 to allow unadsorbed protein to flow from the column. Adsorption was complete when no protein was detected at 280 nm in the effluent. For the isolation of IgG and IgM, the unbound protein eluted with PBS, pH 7.5 was concentrated by ultrafiltration and characterized as described in Appendix E.

Bound protein, as in the case for the isolation of IgA, was eluted from the column by washing with 2.8 KSCN in PBS, pH 7.5 by gravity flow until the A280 of the elucent was less than .05. The adsorbed protein was concentrated by ultrafiltration and dialyzed extensively against barbital-acetate buffer, pH 9.

## APPENDIX H: IMMUNOELECTROPHORESIS

This classical immunoelectrophoretic technique was used for the detection of one or more antigens in an isolated protein fraction.

The immunoelectrophoretic agarose plates were prepared by adding 0.5 g of agarose (Seakem LE, Marine Colloids, Rockland, ME) to 50 ml of tris-barbital buffer, pH 8.6 (containing 22.4 g diethyl barbituric acid (Mallincrodt Chemical Works, St. Louis, MO), 44.3 g Trizma Base (Sigma, St. Louis, MO), .533 g Ca-lactate, and .65 g $NaN_3$ in 1 liter $H_2O$). The agarose beads were allowed to swell in the buffer for 30 minutes after which time the suspension was heated until all the agarose was dissolved. Distilled water was added to replace losses due to evaporation. When the agarose was completely in solution, the agarose solution was cooled to 57 C in a water bath. Twelve ml of the agarose solution was poured by means of a preheated pipette onto Gelbond film agarose support medium (FMC Corporation, Marine Colloids, Rockland, ME) measuring 85x100 mm and 0.2 mm thick. The agarose solution was allowed to harden on a leveled table at room temperature.

Ten microliters of test material were placed into a circular well (4 mm) cut in the agarose gel by a steel cutter. The test material was separated into its components by electrophoresis (LKB Multiphor system, LKB, Gaithersburg, MD) at an applied voltage of 8 v/cm for one hour at 4 C. Five sheets of moistened Whatman #1 filter paper were used for connecting the gel to the electrode vessels. Each electrode vessel contained one liter of tris-barbital buffer, pH 8.6.

After the run of the electrophoresis, one or two longitudinal troughs (40x2 mm) were cut parallel to the electrical flow and at a suitable distance (3 or 4 mm) from the circular well. This was accomplished by the use of a template for immunoelectrophoresis (LKB, Gaithersburg, MD). One hundred microliters of antiserum were placed in the trough. The plate was left for 12-20 hours for diffusion of the antigin and antibody molecules. A precipitin arc was formed by each antigen binding to its corresponding antibody.

After diffusion, the gels were washed, pressed, dried, stained, and destained as described previously (see Appendix C for procedure).

APPENDIX I:   PREPARATION OF ANTISERUM TO CHICKEN
BILE GLOBULINS

Antiserum to chicken bile globulins was prepared in a goat.  The bile
globulin preparation used to immunize the goat (see Appendix F) was
dialyzed in .9% NaCl.  One ml of the bile globulin preparation (5 mg
protein/ml) emulsified in Freunds complete adjuvant (Gibco, Grand Island,
NY) was administered to the goat by subcutaneous injection.  Three booster
injections were given at 10-day intervals.  Seven days after, the last
administration of antigen blood was collected from the goat by jugular
catheter.  The serum was heat inactivated at 37 C for 30 minutes and was
stored at 4 C in .01% $NaN_3$ until used.

## APPENDIX J: ASSAY FOR VITAMIN A IN LIVER

### Sample Storage

The entire liver was taken at necropsy, placed in sealable plastic bags, and stored frozen at -20 C until analysis.

### Sample Extraction

Samples of frozen liver (3-5 g) were weighed, placed in 25x150 mm screw top test tubes and covered with 2.5 g of anhydrous sodium sulfate (J. T. Baker Chemical Co., Phillipsburg, NJ) per gram of liver sample. The liver sample was crushed and mixed with the sodium sulfate. The mixture was covered with 5 ml of chloroform/g of liver sample, sealed with plastic-coated cardboard gaskets and placed in the freezer for 24 hours.

### Carr-Price Reaction

An aliquot of the chloroform extract (1 ml) was placed in a colorimetric tube, and 2.5 ml of 20% antimony trichloride solution (20 g antimony trichloride (Fisher Scientific Co., Fair Lawn, NJ) dissolved in 100 ml chloroform) was quickly and forcefully pipetted into the colorimetric tube. The absorbance at 620 nm was measured at its maximum in a Beckman Spectronic 20 (Bausch & Lomb, Rochester, NY).

### Standard Curve

The amount of vitamin A in the sample was determined from a standard curve ranging from 1 to 10 ug/ml obtained at the same time using reference retinyl acetate (crystalline all-trans retinyl acetate, Eastman Kodak Co., Rochester, NY).

## Aliquots of Chloroform Extracts

Aliquots of 5 ml of the chloroform extracts from the low or no vitamin A groups were placed in colorimetric tubes, evaporated under nitrogen and brought back into solution with 1 ml of chloroform before absorbance was determined.

APPENDIX K:  TABLES

Table K1. Analysis of variance of body weight, feed consumption, and feed efficiency of chicks at 2 weeks of age

| Source | d.f. | Mean squares | | |
| | | Body weight (g) | Feed intake g/day | Feed/ gain |
|---|---|---|---|---|
| Treatment | 2 | 451*** | 5.2 | .03 |
| Retinol 2 vs. retinoic acid 2[a] | 1 | 511*** | 10.4 | .04 |
| Retinol 2 vs. 0.2 | 1 | .162 | 4.4 | .001 |
| Error | 45 | 65 | 3.4 | .02 |

[a]Represents single degree of freedom comparisons between groups.

***$p < .01$.

Table K2. Analysis of variance of body weight, feed consumption, and feed efficiency of chicks at 3 weeks of age

| Source | d.f. | Mean squares | | |
| | | Body weight (g) | Feed intake g/day | Feed/ gain |
|---|---|---|---|---|
| Treatment | 2 | 9708**** | 14 | .159** |
| Retinol 2 vs. retinoic acid 2[a] | 1 | 10379**** | 11 | .087 |
| Retinol 2 vs. 0.2 | 1 | 39 | 0.8 | .030 |
| Error | 45 | 281 | 6 | .022 |

[a]Represents single degree of freedom comparisons between groups.

**,****$p < .05$ and $p < .001$, respectively.

Table K3. Analysis of variance of body weight, feed consumption, and feed efficiency of chicks at 4 weeks of age

| | | Mean squares | | |
|---|---|---|---|---|
| Source | d.f. | Body weight (g) | Feed intake g/day | Feed/ gain |
| Treatment | 2 | 15398**** | 1.06 | .09** |
| Retinol 2 vs. retinoic acid 2[a] | 1 | 11864**** | 1.00 | .08 |
| Retinol 2 vs. 0.2 | 1 | 1160 | .03 | .004 |
| Error | 45 | 604 | 6.86 | .02 |

[a]Represents single degree of freedom comparisons between groups.

**,****p<.05 and p<.001, respectively.

Table K4. Analysis of variance of body weight, feed consumption, and feed efficiency of chicks at 5 weeks of age

| | | Mean squares | | |
|---|---|---|---|---|
| Source | d.f. | Body weight (g) | Feed intake g/day | Feed/ gain |
| Treatment | 2 | 8088*** | 2.85 | .09 |
| Retinol 2 vs. retinoic acid 2[a] | 1 | 204 | 5.63 | .15 |
| Retinol 2 vs. 0.2 | 1 | 7080** | 3.25 | .16 |
| Error | 41 | 1544 | 74 | .06 |

[a]Represents single degree of freedom comparisons between groups.

**,***p<.05 and p<.01, respectively.

Table K5. Analysis of variance of body weight, feed consumption, and feed efficiency of chicks at 6 weeks of age

| Source | d.f. | Mean squares Body weight (g) | Mean squares Feed intake g/day | Mean squares Feed/ gain |
|---|---|---|---|---|
| Treatment | 2 | 21747*** | 215 | .084 |
| Retinol 2 vs. retinoic acid 2[a] | 1 | 9579 | 163 | .161 |
| Retinol 2 vs. 0.2 | 1 | 40898*** | 428** | .111 |
| Error | 41 | 3521 | 76 | .075 |

[a]Represents single degree of freedom comparisons between groups.

**,***p<.05 and p<.01, respectively.

Table K6. Analysis of variance of body weight, feed consumption, and feed efficiency of chicks at 7 weeks of age (Group 0)

| Source | d.f. | Mean squares Body weight (g) | Mean squares Feed intake g/day | Mean squares Feed/ gain |
|---|---|---|---|---|
| Treatment | 2 | 152908*** | 256 | 0.185 |
| Retinol 2 vs. retinoic acid 2[a] | 1 | 50837 | 513 | 0.270 |
| Retinol 2 vs. 0.2 | 1 | 276396*** | 238 | 0.128 |
| Error | 41 | 29437 | 198 | 0.363 |

[a]Represents single degree of freedom comparisons between groups.

***p<.01.

Table K7. Analysis of variance of immunoglobulin and total protein concentrations and hemagglutination inhibition titers of serum of chicks at 2 weeks of age

| Source | d.f. | Mean squares | | | | |
|---|---|---|---|---|---|---|
| | | IgG (mg/ml) | IgM (mg/ml) | IgA (mg/ml) | Total serum protein (mg/ml) | NDV (GMT) |
| Treatment | 2 | .0008 | .14 | .0079 | 16.2 | .79 |
| Retinol 2 vs. retinoic acid 2[a] | 1 | .0002 | .012 | .0056 | 29.7 | 1.56 |
| Retinol 2 vs. 0.2 | 1 | .002 | .25 | .0023 | 1.7 | .25 |
| Error | 21 | .19 | .1 | .0264 | 9.6 | 2.30 |

[a]Represents single degree of freedom comparisons between groups.

Table K8. Analysis of variance of immunoglobulin and total protein concentrations and hemagglutination inhibition titers of serum of chicks at 3 weeks of age

| Source | d.f. | Mean squares | | | | |
|---|---|---|---|---|---|---|
| | | IgG (mg/ml) | IgM (mg/ml) | IgA (mg/ml) | Total serum protein (mg/ml) | NDV (GMT) |
| Treatment | 2 | .015 | .258 | .016 | 13.6 | .292 |
| Retinol 2 vs. retinoic acid 2[a] | 1 | .11 | .47 | .002 | 0.3 | .02 |
| Retinol 2 vs. 0.2 | 1 | 0 | 2.29 | .03 | 18.1 | .04 |
| Error | 21 | .204 | .225 | .02 | 5.1 | 12.8 |

[a]Represents single degree of freedom comparisons between groups.

Table K9. Analysis of variance of immunoglobulin and total protein
concentrations and hemagglutination inhibition titers of
serum of chicks at 4 weeks of age

| Source | d.f. | Mean squares | | | | |
| | | IgG (mg/ml) | IgM (mg/ml) | IgA (mg/ml) | Total serum protein (mg/ml) | NDV (GMT) |
|---|---|---|---|---|---|---|
| Treatment | 2 | .388 | .468* | .167* | 4.9 | 23.29 |
| Retinol 2 vs. retinoic acid 2[a] | 1 | 1.21 | .731* | .160 | 1.1 | 30.25 |
| Retinol 2 vs. 0.2 | 1 | .49 | .672* | .314** | 0.1 | .563 |
| Error | 21 | .625 | .190 | .058 | 9.2 | 26.50 |

[a]Represents single degree of freedom comparisons between groups.

*,**p<.10 and p<.05, respectively.

Table K10. Analysis of variance of immunoglobulin and total protein
concentrations and hemagglutination inhibition titers of
serum of chicks at 5 weeks of age

| Source | d.f. | Mean squares | | | | |
| | | IgG (mg/ml) | IgM (mg/ml) | IgA (mg/ml) | Total serum protein (mg/ml) | NDV (GMT) |
|---|---|---|---|---|---|---|
| Treatment | 2 | 1.49* | 1.91* | .182* | 30.3 | 1.33 |
| Retinol 2 vs. retinoic acid 2[a] | 1 | 2.98** | 3.54** | .028 | 15.1 | 0 |
| Retinol 2 vs. 0.2 | 1 | .081 | .238 | .343 | 15.1 | .67 |
| Error | 9 | .385 | .52 | .064** | 34.4 | 3.0 |

[a]Represents single degree of freedom comparisons between groups.

*,**p<.10 and p<.05, respectively.

Table K11.  Analysis of variance of immunoglobulin and total protein
concentrations and hemagglutination inhibition titers of
serum of chicks at 6 weeks of age

| | | | | Mean squares | | |
|---|---|---|---|---|---|---|
| Source | d.f. | IgG (mg/ml) | IgM (mg/ml) | IgA (mg/ml) | Total serum protein (mg/ml) | NDV (GMT) |
| Treatment | 2 | 3.98 | .605 | .033 | 104 | .17 |
| Retinol 2 vs. retinoic acid 2[a] | 1 | 4.03 | .1128 | .051 | 91 | 0 |
| Retinol 2 vs. 0.2 | 1 | 7.39 | 1.16 | .045 | 21 | .125 |
| Error | 9 | 3.02 | .565 | .082 | 119 | 12.75 |

[a]Represents single degree of freedom comparisons between groups.


Table K12.  Analysis of variance of immunoglobulin and total protein
concentrations and hemagglutination inhibition titers of
serum of chicks at 7 weeks of age (Day 0)

| | | | | Mean squares | | |
|---|---|---|---|---|---|---|
| Source | d.f. | IgG (mg/ml) | IgM (mg/ml) | IgA (mg/ml) | Total serum protein (mg/ml) | NDV (GMT) |
| Treatment | 2 | 1.66** | .500*** | .2336** | 32.4 | 40.58 |
| Retinol 2 vs. retinoic acid 2[a] | 1 | 3.15** | .832*** | .441*** | 32.1 | 66.13 |
| Retinol 2 vs. 0.2 | 1 | 1.56** | .690*** | .222** | 5.4 | 55.13 |
| Error | 9 | 3.08 | .048 | .035 | 66.5 | 65.75 |

[a]Represents single degree of freedom comparisons between groups.

**,***$p < .05$ and $p < .01$, respectively.

Table K13. Analysis of variance of body weight of chicks after
vaccination with Newcastle disease virus–La Sota[a]

| Source | d.f. | Mean squares |
|---|---|---|
| Day 2 After Vitamin A Withdrawal | | |
| Treatment | 2 | .077** |
| Retinoic acid 2 vs. 0.2[b] | 1 | .004 |
| Retinoic acid 2 vs. 0 | 1 | .135** |
| Error | 9 | .013 |
| Day 4 After Vitamin A Withdrawal | | |
| Treatment | 4 | .199**** |
| Retinol 2 vs. Retinoic acid 2 | 1 | .005 |
| Retinol 2 vs. 0.2 | 1 | .12*** |
| Retinoic acid 2 vs. 0.2 | 1 | .031 |
| Retinoic acid 2 vs. 0 | 1 | .536**** |
| Error | 15 | .009 |
| Day 6 After Vitamin A Withdrawal | | |
| Treatment | 2 | .305**** |
| Retinoic acid 2 vs. 0.2 | 1 | .015 |
| Retinoic acid 2 vs. 0 | 1 | .531**** |
| Error | 9 | .019 |

[a]Vaccinations were given 2, 4, or 6 days after vitamin A was
withdrawn from diets.

[b]Represents single degree of freedom comparisons.

**,***,****$p < .05$, $p < .01$, and $p < .001$, respectively.

Table K14. Analysis of variance of immunoglobulin and total protein
concentrations and hemagglutination inhibition titers of
serum of chicks vaccinated 2 days after vitamin A was
withdrawn from diets (Day 2)

| | | Mean squares | | | | |
|---|---|---|---|---|---|---|
| Source | d.f. | IgG (mg/ml) | IgM (mg/ml) | IgA (mg/ml) | Total serum protein (mg/ml) | NDV (GMT) |
| Treatment | 2 | 35.76 | .244 | 4.59* | 18 | 240331**** |
| Retinoic acid 2 vs.0.2[a] | 1 | 18.36 | .061 | .29 | 29 | 35364**** |
| Retinoic acid 2 vs. 0 | 1 | 71.52* | .475 | 8.14* | .11 | 3672**** |
| Error | 9 | 20.51 | .55 | 1.56 | 16 | 12223 |

[a]Represents single degree of freedom comparisons between groups.

*,**** p<.10 and p<.001, respectively.

Table K15. Analysis of variance of immunoglobulin and total protein
concentrations and hemagglutination inhibition titers of
serum of chicks vaccinated 4 days after vitamin A was
withdrawn from diets (Day 4)

| | | Mean squares | | | | |
|---|---|---|---|---|---|---|
| Source | d.f. | IgG (mg/ml) | IgM (mg/ml) | IgA (mg/ml) | Total serum protein (mg/ml) | NDV (GMT) |
| Treatment | 4 | 25.31*** | 8.87*** | 1.46 | 101.2*** | 348488*** |
| Retinol 2 vs. retinoic acid 2[a] | 1 | 21.29** | 1.73*** | 3.41** | 0.775 | 347778** |
| Retinol 2 vs. 0.2 | 1 | 46.56*** | 10.10** | 3.08** | 7.88 | 205441 |
| Retinoic acid 2 vs. 0.2 | 1 | 1.0 | 0.86 | .008 | 41.6 | 423200** |
| Retinoic acid 2 vs. 0 | 1 | 26.21** | 18.30*** | .042 | 270*** | 982802*** |
| Error | 15 | 3.15 | 1.81 | .65 | 10.69 | 58089 |

[a]Represents single degree of freedom comparisons between groups.

**,*** p<.05 and p<.01, respectively.

Table K16. Analysis of variance of immunoglobulin and total protein concentrations and hemagglutination inhibition titers of serum of chicks vaccinated 6 days after vitamin A was withdrawn from diets (Day 6)

| Source | d.f. | Mean squares | | | | |
|--------|------|--------------|-----|-----|-----|-----|
| | | IgG (mg/ml) | IgM (mg/ml) | IgA (mg/ml) | Total serum protein (mg/ml) | NDV (GMT) |
| Treatment | 2 | 63.10** | 14.97 | 1.39 | 27.10 | 576482*** |
| Retinoic acid 2 vs. 0.2 | 1 | 34.36** | .379 | .476 | 3.12 | 736898*** |
| Retinoic acid 2 vs. 0 | 1 | 57.35*** | 25.17* | .938 | 50.0 | 975805*** |
| Error | 9 | 45.41 | 5.66 | 1.30 | 40.14 | 61493 |

[a]Represents single degree of freedom comparisons between groups.

*,**,*** p<.10, p<.05, and p<.01, respectively.

Table K17.  Analysis of variance of IgA, protein, and dry matter concen-
tration in bile of chicks at 7 weeks of age (Day 0)

| Source | d.f. | Mean squares | | |
| | | IgA (mg/ml | Total protein (mg/ml) | Dry matter (%) |
| --- | --- | --- | --- | --- |
| Treatment | 2 | 15* | 7.59 | 0.60 |
| Retinol 2 vs. retinoic acid 2[a] | 1 | 29** | 0.32 | 1.10 |
| Retinol 2 vs. 0.2 | 1 | 3.3 | 13.1 | 0.54 |
| Error | 9 | .45 | 16.74 | 0.77 |

[a]Represents single degree of freedom comparisons.

*,**p<.10 and p<.05, respectively.

Table K18.  Analysis of variance of IgA, protein, and dry matter concen-
tration in bile of chicks vaccinated 2 days after vitamin A
was removed from diets (Day 2)

| Source | d.f. | Mean squares | | |
| | | IgA (mg/ml) | Total protein (mg/ml) | Dry matter (%) |
| --- | --- | --- | --- | --- |
| Treatment | 2 | 267**** | 754 | 7.25* |
| Retinoic acid 2 vs. 0.2[a] | 1 | 31 | 655 | 13.36** |
| Retinoic acid 2 vs. 0 | 1 | 494**** | 1450 | 7.55 |
| Error | 9 | 17 | 442 | 1.96* |

[a]Represents single degree of freedom comparisons.

*,**,****p<.10, p<.05, and p<.001, respectively.

Table K19. Analysis of variance of IgA protein and dry matter concentration in bile of chicks vaccinated 4 days after vitamin A was removed from diets (Day 4)

| | | Mean squares | | |
| Source | d.f. | IgA (mg/ml) | Total protein (mg/ml) | Dry matter (%) |
| --- | --- | --- | --- | --- |
| Treatment | 4 | 152*** | 602 | 3.74* |
| Retinol 2 vs. Retinoic acid 2[a] | 1 | 163** | 371 | 0.21 |
| Retinol 2 vs. 0.2 | 1 | 92** | 454 | 5.10* |
| Retinoic acid 2 vs. 0.2 | 1 | 96** | 1231 | .42 |
| Retinoic acid 2 vs. 0 | 1 | 367**** | 1013 | 8.59** |
| Error | 15 | 20 | 406 | 1.42 |

[a]Represents single degree of freedom comparisons between treatment means.

*,**,***,**** $p < .10$, $p < .05$, $p < .01$, and $p < .001$, respectively.

Table K20. Analysis of variance of IgA, protein, and dry matter concentration in bile of chicks vaccinated 6 days after vitamin A was removed from diets (Day 6)

| | | Mean squares | | |
| Source | d.f. | IgA (mg/ml) | Total protein (mg/ml) | Dry matter (%) |
| --- | --- | --- | --- | --- |
| Treatment | 2 | 647**** | 632**** | 13.4** |
| Retinoic acid 2 vs. 0.2[a] | 1 | 750*** | 1123**** | 6.1 |
| Retinoic acid 2 vs. 0 | 1 | 1150**** | 732**** | 26.8** |
| Error | 9 | 41 | | 3.0 |

[a]Represents single degree of freedom comparisons.

**,***,**** $p < .05$, $p < .01$, and $p < .001$, respectively.

Table K21. Analysis of variance of weight, total protein, IgG, IgM, and IgA concentration of intestine of chicks at 7 weeks of age (Day 0)

| | | | Mean squares | | | |
|---|---|---|---|---|---|---|
| Source | d.f. | Tissue weight (g) | Total intestinal protein (mg/g tissue) | IgG (mg/g tissue) | IgM (mg/g tissue) | IgA (mg/g tissue) |
| Treatment | 2 | .51 | 28 | .02* | .0003 | .0011 |
| Retinol 2 vs. retinoic acid 2[a] | 1 | .87 | 41 | .039** | .00006 | .00003 |
| Retinol 2 vs. 0.2 | 1 | .64 | 42 | .017* | .0005* | .0016 |
| Error | 9 | .28 | 69 | .005 | .0002 | .0028 |

[a]Represents single degree of freedom comparisons.

*,**p<.10 and p<.05, respectively.

Table K22. Analysis of variance of weight, total protein, IgG, IgM, and IgA concentration of intestine of chicks vaccinated 2 days after vitamin A was withdrawn from diets (Day 2)

| Source | d.f. | Mean squares | | | | |
| --- | --- | --- | --- | --- | --- | --- |
| | | Tissue weight (g) | Total protein (mg/g tissue) | IgG (mg/g tissue) | IgM (mg/g tissue) | IgA (mg/g tissue) |
| Treatment | 2 | .507 | 27 | .299** | .007 | .0017 |
| Retinoic acid 2 vs. 0.2[a] | 1 | .639 | 41 | .149 | .00003 | .00017 |
| Retinoic acid 2 vs. 0 | 1 | .865 | 41 | .598** | .009* | .0073 |
| Error | 9 | .284 | 69 | .071 | .003 | .005 |

[a]Represents single degree of freedom comparisons.

*,** $p < .10$ and $p < .05$, respectively.

Table K23. Analysis of variance of weight, protein, IgG, IgM, and IgA concentration of intestine of chicks vaccinated 4 days after vitamin A was withdrawn from diets (Day 4)

| Source | d.f. | Tissue weight (g) | Total intestinal protein (mg/g tissue) | IgG (mg/g tissue) | IgM (mg/g tissue) | IgA (mg/g tissue) |
|---|---|---|---|---|---|---|
| | | | **Mean squares** | | | |
| Treatment | 4 | 1.59*** | 128 | 1.54*** | .126* | .003 |
| Retinol 2 vs. retinoic acid 2[a] | 1 | 1.23** | 18.76 | .232* | .043 | .012 |
| Retinol 2 vs. 0.2 | 1 | .96** | 6.72 | .760*** | .064 | .0017 |
| Retinoic acid 2 vs. 0.2 | 1 | .001 | 12.48 | .031 | .0004 | .006 |
| Retinoic acid 2 vs. 0 | 1 | 1.94*** | 32.76 | 3.42*** | .222** | .005 |
| Error | 15 | .159 | 1044 | .077 | .048 | .009 |

[a]Represents single degree of freedom comparisons.

*,**,*** $p < .10$, $p < .05$, and $p < .01$, respectively.

Table K24. Analysis of variance of weight, protein, IgG, IgM, and IgA concentration of intestine of chicks vaccinated 6 days after vitamin A was withdrawn from diets (Day 6)

| Source | d.f. | Tissue weight (g) | Total intestinal protein (mg/g tissue) | IgG (mg/g tissue) | IgM (mg/g tissue) | IgA (mg/g tissue) |
|---|---|---|---|---|---|---|
| | | | Mean squares | | | |
| Treatment | 2 | 1.63*** | 180 | 7.34* | .258** | .003* |
| Retinoic acid 2 vs. 0.2[a] | 1 | .084 | 192 | 1.42 | .022 | .002 |
| Retinoic acid 2 vs. 0 | 1 | 2.86*** | 321 | 14** | .465** | .005** |
| Error | 9 | .13 | 276 | 1.96 | .051 | .0006 |

[a]Represents single degree of freedom comparisons.

*,**,*** $p < .10$, $p < .05$, and $p < .01$, respectively.

Table K25. Analysis of variance of weight, total protein, IgG, IgM, and IgA concentration of trachea of chicks at 7 weeks of age (Day 0)

| Source | d.f. | Tissue weight (g) | Total protein (mg/g tissue) | IgG (mg/g tissue) | IgM (mg/g tissue) | IgA (mg/g tissue) |
|---|---|---|---|---|---|---|
| | | | | Mean squares | | |
| Treatment | 2 | .015 | .527 | .008** | .0007 | .0026 |
| Retinol 2 vs. retinoic acid 2[a] | 1 | .004 | .316 | .015** | .0008 | .005 |
| Retinol 2 vs. 0.2 | 1 | .011 | 1.05 | .007* | .0012 | .002 |
| Error | 9 | .013 | 5.28 | .002 | .0004 | .007 |

[a]Represents single degree of freedom comparisons.

*,**$p<.10$ and $p<.05$, respectively.

Table K26. Analysis of variance of weight, total protein, IgG, IgM, and IgA concentration of trachea of chicks vaccinated at 2 days after vitamin A was withdrawn from diets (Day 2)

| Source | d.f. | Mean squares | | | | |
|---|---|---|---|---|---|---|
| | | Tissue weight (g) | Total protein (mg/g tissue) | IgG (mg/g tissue) | IgM (mg/g tissue) | IgA (mg/g tissue) |
| Treatment | 2 | .107** | 2.55 | .0134 | .0011 | .003* |
| Retinoic acid 2 vs. 0.2[a] | 1 | .101** | 5.09 | .008 | .0021 | .003* |
| Retinoic acid 2 vs. 0 | 1 | .202*** | 1.04 | .005 | .0004 | .005** |
| Error | 9 | .016 | 1.41 | .0276 | .0015 | .0007 |

[a]Represents single degree of freedom comparisons.

*,**,***p<.10, p<.05, and p<.01, respectively.

Table K27. Analysis of variance of weight, total protein, IgG, IgM, and IgA concentration of trachea of chicks vaccinated at 4 days after vitamin A was withdrawn from diets (Day 4)

| Source | d.f. | Mean squares | | | | |
| --- | --- | --- | --- | --- | --- | --- |
| | | Tissue weight (g) | Total protein (mg/g tissue) | IgG (mg/g tissue) | IgM (mg/g tissue) | IgA (mg/g tissue) |
| Treatment | 4 | .199**** | 22** | .378**** | .0284** | .029** |
| Retinol 2 vs. retinoic acid 2[a] | 1 | .007 | 9 | .038 | .00004 | .003 |
| Retinol 2 vs. 0.2 | 1 | .072* | 11 | .065 | .0345** | .005 |
| Retinoic acid 2 vs. 0.2 | 1 | .128** | 3 | .0008 | .00016 | .017 |
| Retinoic acid 2 vs. 0 | 1 | .500**** | 64*** | .826**** | .0623*** | .041* |
| Error | 9 | .022 | 7 | .041 | .008 | .010 |

[a]Represents single degree of freedom comparisons.

*,**,***,**** $p < .10$, $p < .05$, $p < .01$, and $p < .001$, respectively.

Table K28. Analysis of variance of weight, total protein, IgG, IgM, and IgA concentration of trachea of chicks vaccinated at 6 days after vitamin A was withdrawn from diets (Day 6)

| Source | d.f. | Mean squares | | | | |
| --- | --- | --- | --- | --- | --- | --- |
| | | Tissue weight (g) | Total protein (mg/g tissue) | IgG (mg/g tissue) | IgM (mg/g tissue) | IgA (mg/g tissue) |
| Treatment | 2 | .073 | 3.57 | .728*** | .008 | .004 |
| Retinoic acid 2 vs. 0.2 | 1 | .009 | .208 | .318* | .002 | .002 |
| Retinoic acid 2 vs. 0 | 1 | .135 | 6.28 | 1.45*** | .015 | .008 |
| Error | 9 | .052 | 5.78 | .072 | .008 | .0033 |

[a]Represents single degree of freedom comparisons.

*,***p<.10 and p<.01, respectively.