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Mona H.F. Meleka

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Robert K Ringer
Major professor

Date _____

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THE ROLE OF SELENIUM AND VITAMINS E AND C ON CHICKS INFECTED WITH E.COLI, E. COLI TOXIN OR NEWCASTLE DISEASE

By

Mona H.F. Meleka

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ABSTRACT

THE ROLE OF SELENIUM AND VITAMINS E AND C ON CHICKS INFECTED WITH E.COLI, E. COLI TOXIN OR NEWCASTLE DISEASE

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Five experiments, were conducted to determine the effect of vitamins E and C and selenium (Se) on the performance and immune response of chicks. The chicks were either infected with E.coli or vaccinated with Newcastle disease vaccine. In addition, the susceptibility of chicks to E.coli toxin was evaluated and the resultant lesions were compared to those seen in E.coli infection.

The chicks were fed either a corn-soybean basal diet or the basal diet, supplemented with the nutrients investigated.

Vitamin E/Se deficiency was characterized by nutritional encephalomalacia, exudative diathesis and muscular dystrophy, as well as by lower plasma Se and .tocopherol values. Chicks infected with E.coli had lesions of pericarditis, perihepatitis and airsacculitis.

Supplementing the diet with 300 IU vitamin E reduced mortality, restored weight loss due to E.coli infection and increased antibody titers. The addition of vitamin C reduced mortality due to vitamin E deficiency and had a synergistic effect on antibody titers.

The addition of .3 ppm Se and 150 mg vitamin C in the presence of 50 IU vitamin E and an antioxidant, had an adverse effect on mortality, weight gain and feed intake, but increased the antibody titers.

The chicks were not resistant to E.coli toxin and that the toxin produced lesions comparable to E.coli infection. The addition of 300 IU vitamin E and .2 ppm Se reduced mortality, and increased weight gain and increased antibody titers in chicks infected with E.coli, injected with E.coli toxin as well as in uninfected controls. The addition of 300 IU vitamin E and .2 ppm Se also reduced the weight loss due to vaccination.

In conclusion, the addition of vitamin E and vitamin C or vitamin E and Se reduced the stress due to infection and vaccination while the addition of Se and vitamin C had an adverse effect on chicks.

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INTRODUCTION

The interrelationship between nutrition and infectious diseases has attracted world-wide interest for many years. While interest is high and contribution to improved health is great, specific roles of nutrients to optimum health remains rather obscure, are often complex and do not appear to fit any one pattern. Reliable experimental evidence is hard to obtain, and well controlled experiments encompassing both aspects - infection and nutrition - are difficult to conduct. Departments and units of research are organized to emphasize nutrition or disease and the interrelationship is usually not considered. Much of the research that has been done has been using laboratory animal models, and while this information has supplied useful basic information, the application to man and livestock remains to be established. In addition, working with infectious agents that occur in practical problems requires special facilities to avoid exposure to other animal species including man.

This research was undertaken to evaluate the role of vitamin E, selenium (Se) and vitamin C in colibacillosis and Newcastle disease in poultry. The current literature suggests that these nutrients have an important role in infectious disease. E.coli and Newcastle infection are major problems in the poultry industry. Reducing losses due to these diseases would contribute greatly to the poultry industry and public health.

REVIEW OF LITERATURE

A voluminous amount of literature is available on the role of nutrition in susceptibility to infectious disease. Investigators have observed that nutritional factors contribute either negatively, (Scrimshaw et al., 1968; Squibb and Veros 1961), or positively, (Chandra and Newberne, 1977; Scrimshaw et al. 1968.) to the immune response and disease resistance in experimental animals. Among the nutritional factors affecting immunity and disease resistance are intake of proteins, calories, vitamins and minerals (Beisel et al., 1982; Wilgus, 1980). This literature review concentrates primarily on the more recent role of vitamin E, Se and vitamin C on disease resistance and immune response, especially in poultry.

Disease Resistance and Immune Response:

Vitamin E:

In 1972, Tengerdy et al. reported a significant increase in the immune response of chicks and hens fed a diet enriched with vitamin E. They fed a diet fortified with 60 IU of vitamin E per lb of feed, while the normal diet contained 10 IU of vitamin E per lb. Chicks were immunized at 7 days of age with 0.2 ml of 20% sheep red blood cell suspension. The immune response was measured by the antibody plaque-forming cell test or the hemagglutination (HA) test. In a subsequent experiment,

chicks and hens were fed a diet containing either 6.6, 66 or 132 IU of vitamin E/kg as DL- alpha tocopherol. The supplementation of 132 IU vitamin E/kg resulted in a significant increase in the humoral immune response (Tengerdy and Nockels, 1973).

Marsh et al. (1981) at Cornell fed a diet deficient in vitamin E to two week old chicks and produced reduced antibody titer to sheep red blood cells (SRBC). However, if the chicks were fed diets adequate in vitamin E for the first two weeks and then fed a vitamin E deficient diet, they did not have a depression in antibody response, suggesting that vitamin E may be necessary to the ontogeny of the humoral immune system.

Enhancement of the humoral immune response by vitamin E was also reported in mice fed semisynthetic or natural commercial diets supplemented with 60-180 IU vitamin E/kg (Tengerdy et al. 1973).

In a study by Ellis and Vorhies (1976), pigs were fed a nutritionally complete ration (control ration, CR), CR plus 20,000 IU, (recommended vitamin E) or CR plus 100,000 IU of vitamin E/ton (high E), and injected intramuscularly with Escherichia coli bacterin. Pigs fed the high vitamin E ration developed an anti-E.coli serum antibody titer two to three-fold higher than those fed the unsupplemented diet.

Similar results on the potentiation of immune response by vitamin E were obtained in guinea pigs injected

intraperitonealy with Venezuelan equine encephalitis attenuated live-virus vaccine, (Barber et al. 1977); and in calves and piglets vaccinated with polyvalent E.coli vaccine, (Ballarini et al. 1981). The latter authors noted that the supplementary vitamin E given before vaccination had a suppressing effect on the immune response, but when given at the same time as, or after vaccination it had a stimulatory effect. Sheep vaccinated with Clostridium perfringens type D and fed high amounts of vitamin E gave a stimulatory immune response (Tengerdy et al. 1983). Feeding high amounts of vitamin E (150 and 300 IU/kg) to chicks vaccinated with inactivated Newcastle disease virus resulted in potentiation of the hemagglutination - inhibiting activity, probably by stimulating the production of immunoglobulin G, even though it did not significantly modify the serum hemagglutination inhibition titer (Franchini et al., 1983). Whether or not the stimulatory effect of vitamin E on the immune response is correlated with an increase in resistance to infectious disease has yet to be determined.

Heinzerling et al. (1974b) reported that a dietary supplementation of 180 mg of DL. alpha tocopheryl acetate per kg of diet increased the survival of nonimmunized mice from 20 to 80% when challenged with 20 organisms, and of mice immunized with 0.5 ng of Diplococcus pneumoniae type I polysaccharide from 15 to 70% when challenged with 20,000

organisms. The increased survival time was well correlated with increased specific phagocytosis of the bacteria as well as the nonspecific phagocytosis, as indicated by clearance of carbon particles from blood.

Dietary supplementation of chick diets with vitamin E (150 or 300 mg DL. alpha tocopheryl acetate per kg feed) gave increased protection against a relatively moderate (25-30% mortality) E.coli infection (Heinzerling et al., 1974a). A correlative two to three fold increase in $\log^2$ antibody titer against the E.coli infection indicates that the increased chick survival was in part immunologic. Similar results were reported for turkeys fed diets supplemented with 100 ppm or 300 ppm DL- -tocopheryl acetate and infected with E.coli (Julseth 1974).

The protective immunological role of a combination of vitamin E and vitamin A was evaluated (Tengerdy and Nockels, 1975). Day-old chicks were fed vitamins A or E either separately or in all combinations at the following levels: 0, 150 and 300 I.U. vitamin E and 0, 30,000 and 60,000 I.U. vitamin A/kg diet. Chicks were challenged with E.coli at three weeks of age and HA titers and mortality determined 7 days later. Both primary and secondary HA titers were improved by either vitamin fed alone or in combination. Mortality on the other hand was reduced when either vitamin was given alone but not when given in combination.

Further investigation on the effect of vitamin E and

vitamin A on humoral immunity and phagocytosis in E.coli infected chicks indicated that either vitamin alone increased E.coli clearance and a further improvement was noticed when both vitamins were fed together. The antagonistic effect on mortality was observed again. It was attributed to the fact that high vitamin A caused lower vitamin E content in the liver and spleen (Tengerdy and Brown, 1977).

Chlamydia inoculated lambs, also, appeared to have less extensive pneumonia and quicker recovery manifested itself in greater post-infection feed consumption and heavier weight gains of lambs supplemented with vitamin E (Stephens et al., 1979).

Selenium

Studies evaluating the effects of selenium (Se) indicate similar stimulatory immune responses in several animal species analogous to those observed with vitamin E.

In a series of studies, Spallholz et al. (1973a, 1973b, and 1975) found that supplementation of mouse diets with selenium as sodium selenite enhanced immunoglobulin M and immunoglobulin G antibody titers. They suggested that diets containing 1 to 3 ppm selenium may enhance the immune response to SRBC, and that the enhancement was greatest when Se was administered prior to or simultaneously with SRBC antigen. They reported that Se deficiency or Se toxicity both depressed antibody production (1973c).

In a further study, Spallholz and coworkers (1974), used Swiss-Webster mice and administered tetanus toxoid (TT) vaccine or SRBC antigen simultaneously with different levels of Se and DL-alpha tocopheryl acetate (T). The anti TT, IgM and IgG antibodies were not significantly enhanced in the primary immune response but were enhanced in the secondary immune response. However, SRBC antigen enhanced both primary and secondary immune response. They concluded that the differences in antibody titers were dependent upon the amount of Se and T administered, ratio of Se:T administered as well as the route of administration.

In a different study, weanling C57BL/6J mice were fed 0.0, .1, .5 and 5 ppm Se in a Torula yeast basal diet. There was no significant difference in plaque forming cell response to SRBC among the first generation animals. However, the offspring of the mice fed no Se had a diminished response. The results, thus indicate that different Se diets do not affect the immune response of the first generation animals, but they may affect their offspring (Mulhern et al. 1981).

Desowitz and Barnwell (1980) reported that Swiss-Webster mice can be successfully immunized against malaria (P.berghei) when the vaccine is potentiated by Se, dimethyl dioctadecol bromide (DDA) or both. The cumulative immunoenhancement by Se and adjuvant resulted in increased

survival of mice together with a suppression of the intensity of the parasitemia.

Se was also found to improve the immune response of guinea pigs vaccinated with a polyvalent aluminum hydroxide vaccine against braxy, enterotoxemia, malignant edema and lamb dysentery, (Kadymov and Gaivoronskaya 1982). The response was measured by resistance to challenge infection, blood globulins, and phagocytic activity. Aleksondrowicz (1977) reported that mice fed 50 ug of Se as sodium selenite/kg body weight/day supplementation to a standard ration had increased antibody synthesis to bacterial and mycotic antigens. However, increasing the amount of Se to 2mg/kg body weight/day was not effective in stimulating antibody synthesis.

Shackelford and Martin (1980) also reported a significant increase in anti.SRBC antibody titers of mice fed 1 ppm Se in drinking water. On the other hand mice fed 3 ppm Se in their drinking water had significantly lowered antibody titers. Weanling, Se deficient mice fed a Torula yeast diet had higher mortality than those supplemented with .5 ppm Se as Na_2SeO_3 when infected with Salmonella microorganisms (Serfass et al., 1974). Supplementation of broiler rations with 0.5 to 0.7 mg/kg Se in the form of sodium selenite reduced losses due to perosis, catarrhal enteritis and resulted in improved growth and slaughter quality, (Bershneider et al., 1982).

In research on experimental schistosomiasis in mice, DeWitt (1957) reported that a diet, deficient in Se, tended to diminish the natural resistance of the host and had a profound influence on the survival and development of S. mansoni. Mice fed the deficient diet harbored 69% more parasites than mice fed the control diet, and on microscopic examination of the parasites recovered from mice fed the deficient yeast diet, the somatic development was markedly impaired.

Pigs having high blood glutathione peroxidase (GSH.Px) activity, i.e. a high Se status, had lower susceptibility to diarrhea and pneumonia than those with low GSH.Px values, (Jorgensen and Wegger, 1979). It should be mentioned that the pigs were fed a Se-vitamin E adequate diet, and there were no clinical signs of Se deficiency. This suggests a genetic variation between the animals in their ability to either absorb Se or incorporate it in GSH.Px, resulting in subclinical Se deficiency and lowering the pig's resistance to infections.

Se was also reported to have antiinflammatory properties (Roberts 1963 a. 1963 b.), anticarcinogenic properties and nonspecific immune effects, Spallholz (1981).

Colnago and colleagues (1984a) investigated the effect of Se on peripheral blood leukocytes of chicks infected with Eimeria and reported that Se supplements increased the leukocyte number 11 days post primary infection and at 8 or

24 hours after a challenge infection. This correlates well with the enhanced immune response reported earlier by Spallholz (1973a). They also found that the increase in blood leukocytes about 6 days postinfection with Eimeria was associated with a great increase in the number of neutrophils which are the major phagocytic cells in the population of leukocytes.

Interaction of Vitamin E and Se

Mode of Action:

Influence on Cellular Antioxidant Defense Systems:

Oxidation-reduction reactions, including providing energy, oxidative biosynthesis, biodegradation and detoxification are common and important in biological systems. Oxygen, although required for normal metabolic reaction, can react spontaneously with cellular components resulting in degradation or inactivation of important biological molecules.

Free radicals have been recognized as intermediates of some biological redox reactions important for the maintenance of life. Free radicals and oxygen in the presence of high concentrations of polyunsaturated fatty acids (PUFA), as present in biological membranes, result in oxidative degradation leading to structural changes affecting permeability and function of the membranes. Free radical catalyzed lipid peroxidation can damage membranes

with the release of destructive lysosomal enzymes (Tappel, 1962). The hydroperoxides and secondary products formed, if not removed, may cause irreversible damage to the cells. It is, thus, important to the cell to contain an antioxidant defense system, to act as a free radical scavenger and prevent hydroperoxide accumulation.

Vitamin E and Se are two components found to contribute to antioxidant potential of plasma and tissues (Doni et al., 1984). Fischer et al. (1970) found that erythrocytes from chicks fed a vitamin E deficient diet are more prone to lysis in dilute hydrogen peroxide than red cells from chicks fed a balanced diet.

Lipid peroxidation is very high in the hepatic microsomes of vitamin E-Se deficient chicks, and the membranes have very little protection against peroxidation (Noguchi et al. (1973). Saadat - Noori and Afnan (1970) reported that in the absence of vitamin E, free radicals formed from autoxidation of unsaturated fat, predisposed chicks to infection with latent leukosis virus.

The current mechanism of action of vitamin E and Se as antioxidants was critically reviewed and summarized by Lucy, 1972; Combs et al., 1975; Hoekstra, 1975; Chow, 1979; Sheffy and Schultz, 1979; and VanVleet, 1980.

Influence on Phagocytosis:

Phagocytosis is enhanced by vitamin E. Inert carbon particles were cleared faster from the blood of mice

supplemented with 180 mg of DL alpha tocopheryl acetate per kg diet than unsupplemented mice (Heinzerling et al., 1974b). These researchers also reported increased specific phagocytosis of bacteria when the mice were challenged with Diplococus pneumoniae type I. Tengerdy and Brown (1977) reported that dietary supplementation of the chicken's diet with 300 IU/kg vitamin E significantly increased phagocytosis and antibody production, resulting in reduced mortality caused by E.coli, although neither factor alone gave a significant correlation to mortality.

Tengerdy (1980) later suggested that vitamin E may be stimulating phagocytosis through ubiquinone, since vitamin E injections significantly increased ubiquinone and coenzyme Q_{10}. Conversely, other investigators suggest that the killing of bacteria is partially dependent on peroxides generated through bacterial lipid peroxidation (Shohet and coworkers, 1974). Such peroxidation may be promoted by ubiquinone - a prooxidant, but not by alpha tocopherol an antioxidant. Thus, the phagocytosis enhancing effect of vitamin E may be through increasing the efficiency of some cellular functions to maintain high ubiquinone levels, either by protecting it from oxidation or by being directly involved in its biosynthesis (Folkers, 1974).

Defective microbicidal activity in Se deficient rats was observed by Serfass and Ganther (1975). GSH.Px levels of the depleted group were 1% of the control group. The

phagocytic activity of the polymorphonucleated neutrophils (PMN_s) was not affected by Se deficiency, while the fungicidal activity, as measured by the number of intracellularly killed Candida albicans, was significantly reduced. These results were confirmed by the same authors (1976), in that the superoxide dismutases of PMN_s and alveolar macrophages of the mice were not affected by Se depletion, unlike the Se dependent GSH.Px. GSH.Px activity is known to destroy lipid hydroperoxides which occur in the neutrophil during phagocytosis and are known to inhibit 6 phosphogluconate dehydrogenase and glucose 6-phosphate dehydrogenase. Both enzymes are involved in the production of NADPH, used in oxidative destruction of ingested particles by neutrophils (Johnston, 1978). So the failure to destroy these lipid hydroperoxides in the Se-deficient animals results in defective bactericidal mechanisms of the ingested particles. Similar results were reported by Boyne and Arthur (1979) in Se-deficient steers.

Gyang et al. (1984) at Minnesota compared the phagocytosis and killing of Staphylococcus aureus by PMN in Se-deficient to Se-vitamin E injected dairy cows. There was no significant difference between the two groups in the ability of PMN to ingest bacteria. However, the PMN of the supplemented group killed the bacteria more effectively than the PMN from the deficient cows. Earlier Dietert et al. (1983) noted that dietary deficiency of Se and vitamin E in

chicks resulted in decreased phagocytic activity of both monocytes and heterophils against Salmonella typhymurium. In the monocytes, both the kinetics of phagocytosis and the number of engulfed bacteria/monocyte were affected by the deficiency.

Relation to Prostaglandins:

Prostaglandins are known to inhibit antigen-induced release of histamine, inhibit plaque formation (which is a well established model of humoral antibody response), and inhibit both the ability of human neutrophils to kill the microorganism, Candida albicans, and the postphagocytic extrusion of lysosomal hydrolases of human neutrophils and mouse macrophages. The inhibitory effect of prostaglandins were correlated with the accumulation of intracellular cyclic AMP, (Bourne, 1974). Since the peroxidation of arachidonic acid is an essential early step in the biosynthesis of prostaglandins, vitamin E deficiency may lead to increased prostaglandin biosynthesis. Thus, it appears that vitamin E plays a vital role in this phenomena by antagonizing the peroxidation of arachidonic acid and limiting the entry of precursors into the prostaglandin cascade, limiting prostaglandin biosynthesis, (Sheffy and Schultz 1979). Likoff et al. (1978) determined the effect of supplemental dietary vitamin E and intraperitoneal injection of aspirin on mortality of chicks due to E.coli infection and prostaglandin production in the bursa and

spleen, and found that vitamin E or aspirin significantly decreased mortality and inhibited prostaglandin production. They suggested that vitamin E may enhance the immune response by inhibiting prostaglandin production in the immune organs of the chick. In later work Likoff and colleagues (1981) reported a correlation among increased humoral immunity, phagocytosis and depressed prostaglandin levels in chicks infected with E.coli, but not with decreased mortality. In contrast Chan et al. (1980) reported that vitamin E deficiency caused a considerable depression of prostaglandin cyclooxygenase as indicated by the significant decrease of prostaglandins PGE_2 and PGF_2 production.

Se also was suggested to be involved in modifying the metabolism of arachidonic acid to prostaglandin precursors, thus enhancing the immune response by reducing endogenous production of prostaglandins (Colnago et al., 1984a).

Effect of both Se and vitamin E on the immune response and disease resistance:

Spallholz et al. (1974) concluded that in mice the difference in antibody titer to SRBC and tetanus toxoid were dependent upon the amount of Se, vitamin E or Se:E and the route of administration.

In pigs fed unsupplemented vitamin E-Se practical (cod liver oil supplemented) diets, there was increased morbidity and clinical signs due to swine dysentery. Supplementing

both E/Se improved weight gains, extended incubation period, reduced mortality, days of anorexia and days of hemorrhagic diarrhea (Teige et al. 1977, 1978 and 1982).

The neutralizing antibody titers following vaccination with a canine distemper-infectious hepatitis virus vaccine were lower in dogs fed vitamin E and Se deficient diets than in dogs fed normal diets (Sheffy and Schultz, 1979). Humoral antibody production to SRBC was enhanced in pigs with the supplementation of vitamin E and Se, (Peplowski et al. 1980). The authors also reported a reduction in mortality in young weanling pigs injected with vitamin E/Se at weaning. They concluded that this response may be related to lowering of various environmental stresses.

Marsh and coworkers (1981) reported that deficiencies of E and/or Se in the chick impaired the immune function as measured by hemagglutinating antibody titers. They suggested that both vitamin E and Se are important for optimum immune function in the chicken at two weeks of age, but at three weeks of age Se appeared to replace vitamin E with regard to the immune system.

Jensen and Johnson (1978), reported that vitamin E/Se supplementation resulted in lower mortality and less depression in body weight in experimental coccidial infection in chicks. Similar results were reported by Colnago et al. (1984b). They reported earlier that supplementation of a corn-soy diet with 100 IU vitamin E

and/or 0.25 ppm Se/kg decreased mortality and helped reduce depression due to the pale bird syndrome, (Colnago et al., 1983). Marsh et al. (1982, 1986) suggested that the primary lymphoid organs such as the bursa and thymus, are major targets of Se and vitamin E deficiencies and provide a possible mechanism by which immune function may be impaired.

Vitamin C:

Although it is generally accepted that vitamin C can be synthesized by the bird (Carrick and Hauge, 1925), additional vitamin C improved the growth response of chicks (March and Biely, 1953). The effects of vitamin C on the immune response of birds and other animals, as manifested by antibody production, phagocytosis, and the effect of ascorbic acid on the lymphoid organs (to mention a few) have been summarized (Beisel, 1982).

The effect on the lymphoid organs:

The thymus has been reported to elaborate humoral factors which influence the growth and immunologic function of other peripheral lymphoid organs. Dieter (1969), noted that thymus extracts partially restored weight and hexose monophosphate shunt (HMS) enzyme activity of rat thymus, spleen and lymph node when depressed by x-irradiation. When the thymus extracts were prepared from guinea pigs fed high levels of vitamin C, the regeneration of lymphatic weight and restoring HMS activity was accelerated. Further loss of tissue weight and depressed enzyme activity occurred in the

irradiated rats treated with thymus extract prepared from guinea pigs fed low amounts of vitamin C. This, suggested that thymic humoral factor production or activity is dependent in part on vitamin C. In 1971, the same author reported that vitamin C is not needed for the expression of thymic humoral factor activity, since partial removal of vitamin C from similarly prepared rat thymic humoral factor (THF) preparation did not result in diminished responses, but is involved in some manner with the production of THF during thymic ontogeny.

Effect on Antibody Production:

The role of vitamin C in antibody production in experimental models can be summarized as being controversial. Some workers (Kumar and Axelrod, 1969; Taylor et al., 1978; McCorkle et al., 1980) have not been able to demonstrate a specific role, while others (Long, 1950; Corbel and Wood, 1975; Siegel and Morton, 1977; Anthony et al., 1979; Fraser et al., 1980) obtained evidence that in vitamin C deficiency there is an impairment of cell mediated immunity.

Effect on phagocytosis:

In vitamin C deficient guinea pigs, Nungester and Ames (1948) reported that the exudates were strikingly lacking in white cells and the fragility of those cells present were increased and cells were easily ruptured. The phagocytic

activity was also lowered and varied with the amount of ascorbic acid present in the exudate.

Shilotri (1977a) studied the phagocytic function of leukocytes isolated from ascorbic acid deficient guinea pigs and concluded that the marked decrease in bactericidal activity may be due to reduced particle uptake, as evidenced by impaired glycolytic activity, or to reduced particle destruction as evidenced by impaired phagocytosis induced HMS activity. The enzymes related to bactericidal activities of leukocytes in ascorbic acid deficient guinea pigs were also determined (Shilotri, 1977b). The stimulation in NADPH-oxidase activity under phagocytizing condition was significantly lower in ascorbic acid deficient leukocytes than in control leukocytes. Similarly the extent of release of acid phosphatase from lysozymes during phagocytosis was also low in ascorbic acid deficient leukocytes. The stimulation of the HMS by ascorbic acid was also reported by DeChaletet et al. (1972; 1974), and McCall and DeChaletet (1971). Furthermore, Miller (1969) demonstrated that the combination of hydrogen peroxide and ascorbic acid generates free radicals which kill the bacteria by disturbing the cell wall, thus interfering with selective permeability. A further bactericidal activity is also accomplished by the presence of lysozymes which cause complete disruption of the cell.

On the other hand, Ganguly et al. (1976) noted no significant impairment in phagocytosis of bactericidal cells in vitamin C deficient guinea pigs. The macrophages, were smaller in size and exhibited significantly reduced migration on a glass surface as compared to normal cells. Also, Stankova et al. (1975) found that scorbutic guinea pig neutrophils (PMN) produced hydrogen peroxide (H_2O_2) and killed Staphylococcus aureus as well as PMN, and suggested that ascorbate does not contribute significantly to phagocyte H_2O_2 production or bactericidal killing. Furthermore, Plelsityi and Fomina (1974) reported that prolonged administration of excessive doses of vitamin C to rabbits resulted in a significant decrease in the total bactericidal activity, in serum content of properdin, lysozyme and sharp inhibition of phagocytic activity of the peripheral blood leukocytes.

Effect on disease resistance:

Squibb et al. (1955) noted that coryza infection in New Hampshire chicks of different ages and sexes resulted in significant reduction of serum ascorbic acid. They emphasized the value of nutritional measures in the therapy and prophylaxis of birds infected with coryza. In the same year, Hill and Garren (1955) obtained evidence that ascorbic acid was important in promoting resistance to foul typhoid when fed with high amounts of other vitamins; although alone, it had no effect on resistance. They concluded that

the effect of ascorbic acid might have been due to the antibacterial effect against Salmonella organisms in the intestinal tract of the chick, or due to its antioxidant properties.

Increased amounts of vitamin C did not prevent parainfluenza type III virus infection in cottontopped marmosets, but it did delay the onset of the disease, reduced clinical responses and decreased mortality. The results indicated that ascorbic acid does not involve direct interaction with the virus, but extends its effects on the host, possibly by maintaining cellular and tissue integrity (Murphy et al., 1974).

Relationship to vitamin E and Se:

Perhaps it is not by coincidence that some lesions of vitamin E-Se deficiency also bear resemblance to an ascorbic acid inadequacy. In both deficiencies the integrity of the vascular system is frequently affected with exudative diathesis and/or hemorrhaging as part of the observed pathology.

Vitamin C, like vitamin E and Se, acts as an antioxidant reducing rancidity in the fat of the white rat (Overman, 1942); also, in controlling or preventing free radical reactions (Demopoulos, 1973). Kunert and Tappel, (1983) found that vitamin C reduced *in vivo* lipid peroxidation in guinea pigs, as measured by pentane and ethane production. They also reported that the protection

provided by vitamin C was similar to that provided by reduced glutathione and alpha tocopherol.

Whether vitamin C could affect the severity and frequency of vitamin E deficiency lesions was investigated by Dam et al. (1948). The addition of 0.5% ascorbic acid to the diet delayed and minimized the tendency for exudation. However, there were no conclusions as to its effect on encephalomalacia because of the relatively low incidence of this lesion in chicks. In 1975, Moran et al. reported that adding ascorbic acid to a practical ration deficient in vitamin E and Se substantially reduced associated mortality in growing ducks. The continued appearance of various myopathies but absence of vascular faults supported the research of Caputto et al. (1961), who concluded that the free radical associated compounds resulting from vitamin E and Se deficiency inhibited gulonolactone oxidase and, hence, reduced ascorbic acid formation.

Brown et al. (1974) reported that selenium deficiency in the duck resulted in a marked decline in serum levels of ascorbic acid. They suggested that Se deprivation may interfere with ascorbic acid synthesis. Similarly, vitamin C influenced the metabolism of vitamin E and partially reversed the changes in some of the biochemical parameters resulting from vitamin E deficiency in rats, (Chen and Thacker, 1985). In earlier work, Combs and Scott (1974) had also reported that ascorbic acid significantly reduced the

Se requirement for growth and prevention of exudative diathesis and mortality in vitamin E deficient chicks. They attributed this to an increase in glutathione peroxidase activity, and an increase in the availability and biological utilization of dietary selenium.

Packer et al. (1979) observed a direct free radical interaction between vitamin E and vitamin C, proving the synergistic action reported earlier by Tappel (1968). Tappel had suggested that the two vitamins act synergistically, vitamin E acting as the primary antioxidant and the resulting vitamin E radical then reacting with vitamin C to regenerate vitamin E. These results were confirmed recently by Bascetta et al. (1983), Niki et al. (1984), and Scarpa et al. (1984).

On the other hand, Chen (1981) reported an increase in vitamin E requirement induced by high supplementation of vitamin C in rats. He noted that high supplementation of vitamin C at marginally adequate vitamin E levels significantly increased <u>in vitro</u> erythrocyte hemolysis and liver lipid peroxidation. High vitamin C supplementation also significantly lowered the erythrocyte level of reduced glutathione and plasma level of vitamin E. The addition of vitamin E counteracted these adverse effects, suggesting that vitamin E requirement may be increased with increased vitamin C supplementation.

Escherichia coli Infection

E.coli is a major pathogen of worldwide importance in commercially produced poultry, contributing significantly to economic losses in both turkeys and chicks. In 1980, respiratory infections accounted for 48% of turkey mortality, and colibacillosis accounted for 19% of the respiratory infections (Smith, 1984). In 1985, Clould et al. reported that respiratory diseases annually cost the Delmarva poultry industry more than 8.5 million dollars. Most frequently, respiratory disease is enhanced by a presumably secondary E.coli infection resulting in complicated air sac disease or colibacillosis.

Although E.coli is a normal inhabitant of the lower intestinal tract and is usually present in poultry houses' air, litter and dust, it is able to exploit weakness in the body defenses caused by other infections and environmental, physiological or nutritional stress, resulting in characteristic lesions. Coliform infection has been associated with a variety of conditions in avian species including septicemia, air sacculitis, enteritis, omphalitis, arthritis and coligranuloma (Rosenberger et al. 1985). The multiplicity of diseases occurring in broilers due to E.coli has been reviewed by Gross (1972).

E.coli toxin:

Endotoxins produced by Gram negative microorganisms have been related to many physiological and pathological reactions in a wide range of animals (Thomas, 1954). E.coli endotoxins produce endothelial cell damage and increase pulmonary vascular permeability in man (Meyrick, 1986). It has also been reported to cause the generalized Schwartzman reaction in pigs fed diets low in vitamin E (Tiege, 1977). Since bacterial endotoxins can produce Disseminated Intravascular Coagulation (DIC), and the same reaction could be produced by a vitamin E deficient diet. Whitehair and Miller (1985) questioned whether endotoxins or toxins might be produced as a result of a dietary insufficiency of vitamin E/Se.

While endotoxins may cause lesions in mammals, avian species (chicks and turkeys) appear to be resistant to large doses of endotoxins (Ball et al., 1962; Jordan and Hinshaw, 1964; Cole and Boyd, 1965; Alder and DaMassa, 1978). Conversely, Truscott and Inniss (1967) found that endotoxins prepared by NaCl extraction from certain strains of E.coli were capable of causing lesions and mortality in chicks. The failure to elicit a marked response in the avian species to endotoxins does not prove that endotoxins are not concerned with the pathogenesis of infections due to certain Gram negative microorganisms. Chicks and turkeys are susceptible to infections due to Gram negative

microorganisms, such as Salmonellosis and Pasteurellosis, causing high morbidity and mortality.

Newcastle disease

Newcastle is an infectious, highly contagious and destructive disease of chicks and other birds. The form of the disease varies with the strain of virus. The less virulent form may be nondetectable while other mild forms may induce transitory respiratory illness. Virulent forms could be manifested with severe respiratory, nervous or enteric symptoms. Laying hens usually have lower egg production, with reduced quality or in severe cases a complete cessation of egg production. Vaccination against Newcastle disease (ND) with an attenuated virus has been used as a successful means of protection and control. However, problems continue to occur due to failure to vaccinate properly, failure of the vaccine or existing concurrent and debilitating disease.

SUMMARY

The literature provides considerable evidence that vitamin E, Se and vitamin C have an important role in development of immunity and resistance to infection. Much of this information is from results using laboratory animal models and non-pathogenic antigens. A minimum amount of research has been conducted in poultry using practical-type rations and an infectious agent as encountered in practical poultry operations.

OBJECTIVES

The objectives of this research were:

1. To determine the effect of diets deficient or supplemented with vitamin E or Se on the performance and immune response of chicks infected with a virulent strain of E.coli.
2. To evaluate the interaction of vitamin C with Se and vitamin E on the immune system.
3. To determine the susceptibility of chicks to E.coli endotoxin and the role of vitamin E and Se on the resistance to disease.
4. To determine the effect of vitamin E and Se on the immune response and performance of chicks vaccinated with Newcastle disease vaccine.
5. To obtain training and experience in research on nutrition and disease problems.

MATERIALS AND METHODS

The role of vitamins E and C and Se in susceptibility to E.coli infection and Newcastle vaccination in chicks was investigated in a series of experiments namely:

1. The role of vitamin E and Se on chicks infected with a virulent culture of E.coli.
2. Replication of experiment 1 using a dilution of the E.coli culture.
3. The effect of Se and vitamin C on chicks infected with E.coli.
4. Susceptibility of chicks to E.coli toxins and the role of vitamin E and Se.
5. The effect of vitamin E and Se on chicks vaccinated with Newcastle disease vaccine.

In each experiment the chicks were divided into groups and fed either the basal diet (deficient) or the basal diet supplemented with the nutrients investigated. At two weeks of age, half the chicks fed each diet were given the infectious agent.

I. Chicks and Housing:

One-day-old broiler, Hubbard chicks from Fairview Farm Inc., Remington, Indiana were used. The chicks were wing-banded on arrival. They initially weighed an average of 35 g. They were randomly divided into

groups according to the statistical design given for each experiment. The chicks were housed in raised wire floored (1/2 inch x 1/2 inch welded wire) electrically heated battery brooders. The two batteries were kept in two separate but similar rooms in the Veterinary Research Facilities (Barn F). The battery in one room was used for uninfected controls while the other was used for the infected birds. The health status was checked daily and any mortality recorded. Feed intake and weight gain were monitored weekly. All chicks that died during the experiment or were killed at the end of the trial were necropsied and postmortem lesions recorded.

II. Diets:

The chicks were fed an oil-supplemented commercial corn-soybean meal diet, (ingredients and percentages are listed in tables 1 and 2). Feed and water were provided ad libitum.

The diets were prepared with the help of Dr. E. R. Miller at the Swine Research Center. The diet's composition was as described by Tengerdy and Nockels (1973) with few modifications. The fish oil was used to deplete the chicks' reserves of vitamin E, as this oil had produced typical vitamin E deficiency signs when fed to chicks (National Research Council, 1971).

Table (1) Composition and Calculated Nutrient Analysis of Diets Used in experiments 1,2,4 and 5.

Ingredient	%
Corn	35.71
Soybean meal 44%	49.0
Cod liver oil	10.0
Ground limestone	0.94
Dicalcium phosphate	3.18
Iodized salt	0.58
Methionine	0.27
Mineral mix (1)	0.06
Vitamin mix (2)	0.26
Total	100.00

(1) Mineral Mix supplies/kg diet: 50 mg manganese, 50 mg iron, 5 mg copper, 0.5 mg cobalt, 1.5 mg iodine, 50 mg zinc and 0.15 mg selenium.

(2) Vitamin Mix supplies/kg diet: 10,000 IU vitamin A, 880 IU vitamin D, 21.5 mg niacin, 5.5 mg pantothenic acid, 4.4 mg riboflavin, 2.9 mg menadione, 440 mg choline, 1.5 mg folic acid, 2.3 mg thiamin, 6.6 mg pyridoxine and 10.3 mcg vitamin B_{12}.

Calculated Nutrient Analysis:

Protein, % 26.6 Calcium, % 1.26 Lysine, % 1.49

ME, kcal/kg 3146 Phosphorus, % .75

Table (2) Composition and Calculated Nutrient Analysis of Diet Used in experiment 3 :

Ingredient	%
Corn	54.03
Soybean meal 44%	39.70
Corn oil	2.03
Dicalcium phosphate	1.80
Ground limestone	1.11
Salt	.25
Vitamin mix (1)	.26
Mineral mix (2)	.05
Methionine	.27
Ethoxyquin	.50
Total	100.00

(1) Vitamin mix supplies/kg diet: 10,000 IU vitamin A, 1000 IU vitamin D_3, 50 mg vitamin E, 2.5 mg menadione, 2.3 mg thiamin, 4.4 mg riboflavin, 12 mg pantothenic acid, 35 mg niacin, 5 mg pyridoxine, 1.2 mg folic acid, 1300 mg choline, and .01 mg B_{12}.

(2) Mineral mix supplies/kg diet: 60 mg manganese, 80 mg iron, 5 mg copper, 50 mg zinc, .5 mg iodine, .5 mg cobalt.

Calculated Analysis

ME, kcal/kg	3000.00
Protein, %	23.50
Calcium, %	0.91
Phosphorus, %	0.57

III. Infectious Agents:

A. E.coli:

E.coli strain 078:T_3 isolated from a natural outbreak of airsacculitis and grown on agarslant,

was kindly provided by Dr. Saif, Wooster, Ohio. The strain 078 has been reported to be of high pathogenicity to chicks (Rosenberger et al. 1985). The microorganism was cultured in trypticase soy broth and incubated at 37°C for 18-20 hours, then it was transferred to trypticase soyagar slants.

- BBL trypticase soy broth and agar were prepared according to the manufacturer's instructions (BBL Microbiology systems, Becton Dickinson and Co., Cockeysville, MD 20130).
- Cultures were incubated in Stabil Therm Dry type Bacterial Incubator (Blue M electric company, Blue Island, ILL. USA)

1. Bacterial Count:

   To determine the number of microorganisms/ml culture for exposure. The procedure of Benson, 1977 was followed, (Appendix A.1.)

2. Bacteriological Examination:

   a. Sterile swabs from pericardial sacs, lungs and livers of birds that died after E.coli infection were cultured in trypticase soy broth and agar to examine E.coli colonies and to determine the presence of any other bacterial contamination.

   b. Smears from the heart and liver were

stained by Gram stain and examined microscopically for the detection of E.coli microorganisms. For staining procedures refer to Appendix (A.2. and A.3.).

Some of the chicks that died due to infection were sent to the Animal Health Diagnostic Labratory for confirmation of the bacteriological results.

B. E.coli toxins:

1. Purified Endotoxins:

a. Purified endotoxin (10 vials of 10 mg each) were purchased form Difco Lab (Detroit, Michigan). According to the company, the Lipopolysaccharide was prepared from E.coli strain 055:B_5 by phenol-water extraction method of Westphal.

b. Purified endotoxin (150 mg) was prepared from strain 0155:B_5 provided by Dr. Wilson, Pennsylvania State University. This endotoxin was also extracted by phenol-water extraction method of Westphal. The endotoxins were diluted in phosphate buffered saline before injection.

2. Crude Toxin:

This toxin was prepared by the salt extraction method described by Truscott and Inniss (1967).

- E.coli strain 078:T_3 was cultured in broth for 18-20 hours.
- .1 ml was then inoculated on agar plates and further incubated for 18-20 hours.
- The colonies were harvested in distilled water and washed 3 times using HNS centrifuge at 1500 rpm. (International Equipment Co., Needham Heights, Nass. USA.)
- Portions of the washed cells (1.5 g dry weight) were suspended in 50 ml of 9% NaCl and continuously mixed at room temperature for 30 minutes and then centrifuged at 37,000 x g for 20 minutes.
- The super-natant was dialyzed for 10 hours at 5C with frequent changes of water.
- The crude toxin extract was administered to chicks intravenously.

C. Newcastle Vaccine:

The B_1 type, Lasota strain LV, CEO Clonevac-30 was provided by Dr. Saif at the Ohio Agricultural Research and Development Center, Wooster, Ohio. It was the attenuated virus used to vaccinate chicks. The vaccine

was diluted in 500 ml phosphate buffered saline and injected I/M in the chicks as suggested by the manufacturer (International Biologics, Inc. Millsboro, Delaware, USA).

IV. Collection of Samples:

A. Blood:

Blood samples were collected from the brachial vein one week before infection, and 1, 2 and 3 weeks postinfection (except for the Newcastle disease experiment). The samples were allowed to clot and the separated serum was frozen at -20C and used later for determination of antibody titers. At the end of each experiment blood samples were collected into tubes containing heparin from randomly chosen chicks in each group. They were then centrifuged and the plasma was frozen for Se and alpha tocopherol analysis later.

B. Tissues:

Muscle, brain, skin and adipose tissue were collected from birds that died due to dietary deficiency and from control birds for histopathological examination. Also liver, lung, kidney, intestines and spleen tissue were collected from infected as well as control birds for histopathologic examination. The specimens

were fixed in 10% neutral buffered formalin.

V. Laboratory Analysis:

A. Selenium

Plasma samples were analyzed for selenium in the Animal Science Comparative Nutrition Laboratory with the advice of Phylis Whetter using the spectrofluorometric technique described by Whetter and Ullrey (1978). The samples were analyzed in duplicates, digested with HNO_3, extracted with cyclohexane, values read on a spectrofluorometer, and calculated on a curvilinear regression. For detailed procedure refer to Appendix (B.1.).

B. Alpha tocopherol

A total lipid extract from 1 ml plasma and containing internal standards of DL. alpha tocopheryl acetate was injected onto a high pressure liquid chromatograph with a reverse phase column developed with methanol-water. An ultraviolet detector with 280-nm filter was used, as described by Bieri et al., (1979). The tocopherol is quantitated by the peak height ratio method, (Appendix B.2.).

C. Antibodies:

1. To test the immunity produced by E.coli, the passive hemagglutination titer of serum against a purified lipopoly-saccharide

antigen of E.coli strain was used (Herbert, 1967; Neter et al., 1956). For details see Appendix (B.3.a.)

2. Hemagglutination - inhibition:

The antibody titers of birds vaccinated with ND vaccine were determined by microtest procedure for determining Newcastle hemagglutination-inhibition (HI) antibody titers, (Beard and Wilkes, 1973). The procedure is described in Appendix (B.3.b.).

VI. Histopathological Techniques

Tissues selected at necropsy for microscopic examination were processed in cooperation with the Pathology Department Histopathology Laboratory according to the technique of Luna, 1986. They were fixed in 10% neutral buffered formalin, prepared in paraffin blocks, cut at 6 um thickness and stained with hematoxylin and eosin. Refer to Appendix (c) for details.

VII. Statistical Analysis:

Feed intake, weight gain and $\log_2$ antibody titers were analyzed using analysis of variance as described by Gill(1981). Chi square (Gill, 1981) was used to analyze data for mortality rate. Only results with $P<0.1$, using Student's t test was considered significant. Tables (3 to 6) give the source of variation and degrees of

freedom (df) for all experiments.

Table (3) Source of Variation and Degrees of Freedom Experiments 1 and 2,

Source of Variation	df
Diets (D)	3
Infection (I)	1
D x I	3
Error (1)	16
Time (T)	4
D x T	12
I x T	4
D x T x I	12
Error (2)	64

Table (4) Source of Variation and Degrees of Freedom Experiment 3

Source of Variation	df
Diets (D)	3
Infection (I)	1
D x I	3
Error (1)	16
Time (T)	4
D x T	12
I x T	4
D x T x I	12
Error (2)	64

Table (5) Source of Variation and Degrees of Freedom Experiment 4

Source of Variation	df
Diet (D)	1
Infection (I)	2
D x I	2
Error (1)	12
Time (T)	4
D x T	4
I x T	8
D x I x T	8
Error (2)	48

Table (6) Source of Variation and Degrees of Freedom Experiment 5

Source of Variation	df
Diets (D)	1
Infection (I)	1
D x I	1
Error (1)	8
Time (T)	2
D x T	2
I x T	2
D x T x I	2
Error (2)	16

RESULTS

Experiment I

In this experiment the effect of vitamin E and C, singly or in combination on the performance and immune response of chicks infected with E.coli was determined. The 120 day-old broiler chicks were divided, according to a 4 x 2 factorial experiment, into eight groups with 15 chicks each. Each group was further divided into three replicates of five chicks each.

They were fed the basal diet, (Table 1), that contained 10% cod liver oil or basal diet with the addition of 300 IU alpha tocopherol or 150 mg vitamin C/kg diet or both. At three weeks of age, one-half of the chicks fed each diet were injected in the posterior thoracic air sac with 0.15 ml of E.coli suspension containing 1.4 x 10^9 micro organisms/ml.

General:

Some of the chicks fed the basal diet started developing clinical signs of vitamin E deficiency after two weeks. Nutritional encephalomalacia was manifested in ataxia, incoordination and spasms of the limb muscle followed by complete prostration (Figure 1). The chicks that developed deficiency signs were unable to eat and died within a week.

Weight Gain:

Supplementation of the basal diet with 300 IU vitamin E/kg or both vitamins significantly increased weight gain in the second week ($P<.025$) and the third week ($P<.0005$), (Table 7). Addition of 150 mg vitamin C/kg diet to vitamin E supplemented diet caused a significant ($P<0.1$) increase in weight gain during the second and third week; while the addition of vitamin C to the vitamin E deficient diet produced a nonsignificant increase in weight gain during the second week and a significant ($P<.0005$) increase in weight gain by the third week.

Table (7) The Effect of Vitamin E and Vitamin C on Weight Gain of Chicks Before Infection (gm/bird/day)

Time Weeks / Diet	1	2	3
-E	9.68	24.72[a]	27.82[a]
+E	10.25	26.81[b]	34.34[b]
-E+C	9.58	25.48[a]	33.11[b]
+E+C	10.84	28.32[c]	35.71[c]

SEM 0.723
Number of birds in each group 30.
[abc] Different superscripts in same column are significantly different ($P<.1$)

Feed Intake:

Chicks fed the basal diet supplemented with 300 IU vitamin E had a nonsignificant increase in feed intake at one and two weeks and a significant ($P<0.025$) increase in feed intake during the third week, (Table 8). Addition of both vitamins to the basal diet resulted in a significant ($P<.1-.025$) increase in feed intake at the second and the third week, as with weight gain.

Table (8) The Effect of Vitamin E and Vitamin C on Feed Intake of Chicks Before Infection (gm/bird/day)

Time Weeks / Diet	1	2	3
Basal	12.73	34.29[a]	49.58[a]
Basal + E	16.40	37.27	55.47[b]
Basal + C	14.13	36.78	51.70[a]
Basal + C + E	14.57	39.11[b]	57.72[b]

SEM 1.769
Number birds in each group 30 birds.
[abc] Different superscripts are significantly different ($P<0.025$)

Mortality rate:

Chicks fed the basal diet supplemented with 300 IU vitamin E/kg had a significant reduction in mortality, (Table 9). The addition of 150 mg vitamin C/kg to the basal diet caused a significant ($P<.1$) decrease in mortality and the addition of vitamin C to the supplemented diet resulted in a further decrease in mortality rate.

The chicks infected with E.coli had a high mortality rate (90%) during the first three days after infection regardless of the diet. This was believed to be due to the high virulence of the microorganism. Hence, it was difficult to assess the effect of diet on the mortality rate due to infection.

Table (9) The Effect of Vitamin E and Vitamin C on the Mortality Rate of Chicks Before Infection

Diets	Total # of Birds	# Surviving	# Dead	% Mortality
Basal (B)	30	25	5	16.64
B + 300 IU E	30	29	1	3.33
B + 150 mg C	30	27	3	10.00
B + 300 IU E + 150 mg C	30	30	0	0.00

Gross Lesions and Histopathological Examination:

The chicks that died due to the deficiency of vitamin E had hemorrhages in the cerebellum.

On histopathological examination vacuolation and degeneration in the granular layer of the cerebellum, together with congestion and pyknosis of the neurons were present, (Figure, 2).

The chicks that died due to E.coli infection had lesions of acute septicemia with advanced vascular congestion. There was myopathy and necrosis in the breast muscles. Livers were dark and swollen. Many chicks developed pericarditis, perihepatitis and airsacculitis, (Figure 3). There was enteritis with hemorrhages along the intestinal tract. The lungs were solidly hepatized and congested

Microscopically there was a separation of muscle fibers and sarcolemma in the heart, and cellular proliferation due to pericarditis. E.coli microorganisms were detected in the blood vessels.

The proventriculus was inflammed, congested, with hemorrhage and edema between the glands. The spleen was also congested with an increase in the reticulo-endothelial cells. The liver was congested with little necrosis. There was sloughing of the cells in the bursa of Fabricus.

Bacterial examination:

E.coli microorganisms were isolated from the liver, ascitical fluids and pericardial fluids. No other bacterial contamination was present.

Figure 1. Nutritional encephalomalacia in vitamin E deficient chicks.

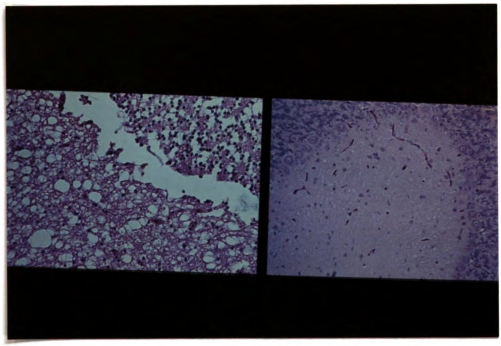

Figure 2. Microscopic appearance of brain of chick. Hematoxylin and eosin.
Left : Nutritional encephalomalacia in vitamin E deficient chick. Vacuolation and pyknosis of purkinji fibers.(25X)
Right : Control fed 300 IU vitamin E.

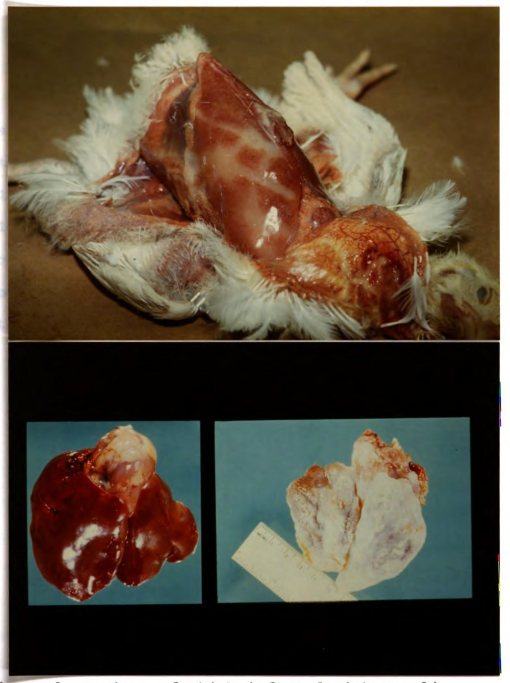

Figure 3. Lesions of chick infected with E.coli.
Above : Myopathy of breast muscle and congestion.
Lower left : Heart and liver of uninfected chick.
Lower right : Heart and liver of E.coli infected chick with pericarditis and perihepatitis.

Experiment 2

Experiment 2 was a replicate of experiment 1 using the same number of chicks, statistical design and diets. Due to the high mortality rate after E.coli injection in experiment 1, the E.coli suspension used in experiment 1 was diluted 1:10 - 1:100,000, and five groups of eight chicks each (two control birds from each dietary group) were injected in the posterior thoracic air sac with 0.15 ml. The lesions and the mortality % are given in Table (10).

Table (10) Postmortem Lesions and Mortality % of Four Week Old Chicks Injected with Different Dilutions of E.coli Suspension:

Dilution of E.coli Suspension	Lesions	Mortality %
1:10	pericarditis, perihepatitis, airsacculitis and edema in 3 birds of the 4 dead ones	50
1:100	pericarditis, airsacculitis and slight perihepatitis in the 2 dead birds	25
1:1000	pericarditis, airsacculitis and slight perihepatitis in the 2 dead birds	25
1:10,000	No lesions	25
1:100,000	No lesions	25

Chicks were injected at two weeks of age with E.coli suspension diluted 1:1000, which resulted, in general, in less mortality.

General:

The chicks fed the deficient diet had signs of vitamin E deficiency as in experiment 1, and also had lower plasma alpha tocopherol values at the end of the experiment (five weeks), (Table 11).

Table (11) Plasma alpha tocopherol of Chicks Fed Diets Deficient or Supplemented with Vitamin E, C or both and Infected or Not Infected with E.coli:

Infection	not infected	infected
Diet	ug alpha tocopherol/ml plasma	
Basal (-E)	.02 ± 0 (2)	.105 ± 0.06 (2)
Basal + E	1.46 ± 0.678(2)	2.05 ± 0.636(2)
Basal + C	0.325 ± 0.318(2)	.125 ± 0.105(2)
Basal + E + C	3.25 ± 0.035(2)	2.24 ± 0.12 (2)

Mean ± Std dev.
( ) Number of Samples Analyzed

Weight Gains:

In experiment 2 the addition of either 300 IU vitamin E or 150 mg vitamin C/kg diet or both did not cause any significant improvement in weight in the uninfected control group, (Table 12).

Infection reduced ($P<.1$) weight gains as compared to the uninfected control chicks fed diets supplemented with vitamin E or vitamin C one week after infection. Infection also reduced weight gain ($P<.1$) in the chicks fed the basal diet two and three weeks postinfection. However, the chicks fed the basal diet supplemented with both vitamins did not have significant difference in weight gain from the uninfected controls.

Within the infected group, the addition of both vitamins to the basal diet significantly ($P<.1$) increased weight gain over the other three diets one week after infection. Also, addition of both vitamins significantly increased weight gain over the basal diet ($P<.005$) and the basal diet and vitamin C ($P<.025$), two weeks after infection. Three weeks after infection the addition of both vitamins still resulted in higher weight gain than the basal diet ($P<.025$), basal diet and vitamin E ($P<.1$) and the basal diet and vitamin C ($P<.05$).

Table (12) The Effect of Vitamin E and Vitamin C on Weight Gains of Chicks Infected with E.coli (gm/bird/day)

Infection	Noninfected					Infected				
Time Wks / Diet	1	2	3	4	5	1	2*	3	4	5
Basal (-E)	15.09	27.77	34.74	43.92	44.82	17.52	28.11	30.46	33.52	35.95
Basal + E	14.54	24.24	39.75	49.75	47.10	16.52	27.28	26.44	54.52	40.44
Basal + C	16.89	29.13	37.89	43.24	40.79	16.70	27.98	30.88	37.34	38.42
Basal + E + C	16.15	28.95	33.28	44.70	46.49	15.08	26.87	39.06	47.95	47.74

SEM 3.60

Number of birds in each group 15.

* chicks were infected at the end of two weeks.

Feed Intake:

In this experiment there was no significant difference between dietary groups in feed intake during the first two weeks in the noninfected group, (Table 13).

On the third week, addition of vitamin C to the basal diet increased ($P<.025$) feed intake. Also the chicks fed vitamin C had higher ($P<.05$) feed intake than those fed vitamin E. During the fourth week, still the chicks fed vitamin C had higher intake than those fed vitamin E ($P<.1$). On the fifth week the supplementation of the basal diet with vitamin C increased feed intake ($P<.1$); also, the chicks fed the vitamin C supplemented diet had higher intake than those fed vitamin E ($P<.025$) or both vitamins ($P<.05$).

Infection reduced feed intake in chicks fed all diets in comparison to uninfected chicks ($P<.005$). Two and three weeks after infection, the chicks fed the basal diet and those supplemented with vitamin C still had lower intake ($P<.05$, $P<.025$) than uninfected controls, while there was no significant difference in feed intake in groups fed vitamin E or both vitamins.

Within the infected group there was no significant difference between the chicks fed the four diets during the first two weeks. During the third week, addition of both vitamins resulted in higher feed intake ($P<.1$) than the basal diet. While on the fourth week, there was no significant difference. On the fifth week the addition of

both vitamins still increased feed intake ($P<.01$) over that of the basal and vitamin E supplemented diets ($P<.1$) and the addition of vitamin C resulted in higher feed intake than that of the basal diet ($P<.1$).

Table (13) The Effect of Vitamin E and Vitamin C on Feed Intake

Table (13) The Effect of Vitamin E and Vitamin C on Feed Intake of Chicks Infected with E.coli (gm/bird/day)

Infection	Noninfected					Infected				
Time Wks / Diet	1	2	3	4	5	1	2*	3	4	5
Basal (-E)	17.83	38.63	54.13	86.50	85.77	18.03	41.17	33.27	72.57	70.93
Basal + E	14.93	36.80	56.97	81.33	79.77	17.20	46.20	39.13	82.13	79.70
Basal + C	19.20	41.17	71.47	95.13	97.60	18.33	40.47	43.70	77.10	82.50
Basal + E + C	18.77	43.57	61.63	88.03	82.00	18.00	41.20	46.87	80.63	90.97

SEM 5.81

Number of birds in each group 15.

* The chicks were infected at the end of two weeks.

Mortality:

In the noninfected group, supplementing the diet with 300 IU vitamin E reduced ($P<0.01$) mortality rate, (Table 14). Also the addition of vitamin C to the basal diet or the addition of both vitamins significantly ($P<0.01$) lowered the mortality rate. Infection caused a significant ($P<0.001$) increase in the mortality rate. There was no significant difference between the dietary groups in mortality rate after infection.

Gross Lesions and Histopathology

The chicks fed the deficient diet had clinical signs and lesions of vitamin E deficiency as described in experiment 1. Lesions due to E.coli infection, histopathological findings and bacteriological examination were as in experiment 1.

Table (14) The Effect of Vitamin E and Vitamin C on the Mortality Rate of Chicks Infected with E.coli:

	Not Infected				Infected (1)			
Diet (2)	Total # of Chicks	Number Surviving	Number Dead	Mortality %	Total # of Chicks	Number Surviving	Number Dead	Mortality %
Basal	15	11	4	26.67	15	8	7	46.67
Basal + E	18	16	2	11.11	15	10	5	33.33
Basal + C	15	14	1	6.67	15	8	7	46.67
Basal + E + C	15	14	1	6.67	15	6	9	60.00

(1) Infection significantly P<.001 increased mortality rate.

(2) Supplementing the diet with 300 IU vitamin E resulted in significantly lower (P<.01) mortality rate.

Supplementing the basal diet with vitamin C (150 mg/kg) or both vitamins significantly (P<.01) lowered the mortality rate.

Antibody titers:

The addition of vitamin E to the basal diet significantly ($P<.1$) increased antibody titer one week after infection, and the addition of both vitamins resulted in an additional increase ($P<.0005$), (Table 15). Chicks fed the basal diet with vitamin C had a lower ($P<.01$) antibody titer than those fed the basal diet supplemented with vitamin E.

Two weeks after infection, supplementation of the basal diet with vitamin E increased antibody titer ($P<.005$); and the supplementation with both vitamins resulted in a still higher antibody titer ($P<.005$). When vitamin C was added to the basal diet, there was a lower ($P<.005$) antibody titer than when vitamin E was added.

On the third week after infection, addition of either vitamins alone significantly increased ($P<.05$) antibody titers over that of the basal diet or the addition of both vitamins.

Table (15) The Effect of Vitamin E and Vitamin C on the Immune Response of Chicks as Measured by Hemagglutination:

Log_2 titer

	Week After Infection		
Time / Diet	1	2	3
Basal	1.76 ± .69 (7)	2.436 ± .51(7)	1.99 ± .00(5)
Basal + E	2.159 ± .288(7)	3.322 ± .76(9)	2.49 ± .49(8)
Basal + C	1.493 ± .48 (5)	2.489 ± .86(5)	2.934 ± 1.40(4)
Basal + E + C	3.123 ± .131(5)	3.82 ± 2.24(4)	1.99 ± 0.0 (5)

( ) Number of birds
Mean ± Std. dev.

Experiment 3

In experiment 2, vitamin C reduced mortality due to vitamin E deficiency and had a stimulatory effect on the immune response when added to vitamin E. Due to the close relationship of vitamin E and Se, this experiment was conducted to determine whether the addition of vitamin C to a diet unsupplemented or supplemented with Se would have the same stimulatory effect on the immune system.

The 120 chicks were divided into eight groups according to a 2 x 4 factorial design. Each group was further divided into three replicates of five chicks each. The chicks were fed the basal diet, (Table 2), basal diet with 0.3 ppm Se, with 150 mg/kg vitamin C or with 0.3 ppm Se and 150 mg/kg vitamin C. The chicks were infected at two weeks as in experiment 2.

General:

The chicks fed the basal diet did not develop any clinical signs of exudative diathesis or muscular dystrophy. Also, the chicks that died in this group did not have any atrophy or changes in the pancreas. Chicks fed the basal diet had lower plasma Se values than those supplemented with Se, (Table 16).

Table (16) The Effect of Se, Vitamin C and E.coli Infection on Plasma Se Values:

Diet	Infection	Se(ug/ml plasma)
Basal	-	.047 ± .007 (8)
	+	.038 ± .004 (8)
Basal + Se	-	.134 ± .007 (8)
	+	.128 ± .015 (8)
Basal + C	-	.037 ± .005 (8)
	+	.043 ± .019 (8)
Basal + C + Se	-	.128 ± .011 (8)
	+	.170 ± .050 (8)

Mean ± Std. Dev.

( ) Number of Samples

Weight Gains:

The weight changes are summarized in Table (17). During the first two weeks there was no significant difference in weight gains of chicks fed the four diets. On the third week the chicks fed the basal diet had higher weight gain than those fed the diet supplemented with 0.3 ppm Se ($P<.025$), those supplemented with 150 mg vitamin C ($P<.1$) and those supplemented with both Se and vitamin C ($P<.005$) in the uninfected group. On the fourth week the basal diet still resulted in more gain than the diet

supplemented with 0.3 ppm Se, the diet supplemented with 150 mg vitamin C, and the diet supplemented with both Se and vitamin C ($P<.1$ - $P<.025$). Also, the chicks fed the diet supplemented with Se had higher weight gain than those supplemented with both Se and vitamin C ($P<.1$).

At five weeks, the chicks fed the basal diet still had higher weight gain than chicks fed the diet supplemented with .3 ppm Se ($P<.01$), those supplemented with 150 mg vitamin C ($P<.005$) or those supplemented with both ($P<.0005$) in the uninfected group.

Infection caused a significant reduction in weight gain when compared to uninfected controls one week after infection in chicks fed the basal diet ($P<.0005$), those fed diets supplemented with Se ($P<.025$) and those fed diets supplemented with vitamin C ($P<.005$).

Two weeks after infection the infected chicks still had lower gains than the control chicks, in the group fed the diet supplemented with Se, while the chicks fed the basal diet and those supplemented with vitamin C or both Se and vitamin C did not differ significantly from uninfected controls. On the third week after infection the chicks fed the basal diet or those fed the diet supplemented with Se or vitamin C had significantly lower gain than the uninfected controls ($P<.025$ - $P<.0005$). The chicks fed diets supplemented with both Se and vitamin C had no significant difference from uninfected controls.

Within the infected group, there was no significant difference in weight gain between chicks fed the four diets during the first three weeks. During the fourth week, the chicks fed the basal diet had higher weight gain than those fed the diet supplemented with 0.3 ppm Se or 150 mg vitamin C. On the fifth week, the infected chicks fed the basal diet and those supplemented with .3 ppm Se had higher weight gain than those fed the diet supplemented with both Se and vitamin C ($P<.1$).

Table (17) The Effect of Vitamin C, Se and E.coli Infection on Weight Gains of Chicks

Infection	Not Infected					Infected				
Time Wks / Diet	1	2	3	4	5	1	2*	3	4	5
Basal	10.64	15.60	29.39	37.74	48.71	12.03	16.26	13.55	33.25	28.71
Basal + Se	11.15	14.01	21.03	31.71	38.93	13.63	14.26	12.73	24.98	30.16
Basal + C	11.53	14.23	23.04	28.31	37.74	12.50	17.19	12.23	28.00	25.22
Basal + Se + C	13.10	17.30	17.49	25.68	24.86	13.21	15.13	13.59	29.52	22.62

SEM 2.71

Number of Birds in each group 15.

The chicks were infected at two weeks of age.

Feed Intake:

In this experiment there was no significant difference in feed intake in chicks fed the four diets during the first three weeks, (Table 18). On the fourth week, still there was no significant difference in feed intake, in the uninfected group between the chicks fed the basal diet and those fed diets supplemented with Se. The chicks fed the basal diet had higher intake than the chicks fed diets supplemented with vitamin C ($P<.05$) and those fed diets supplemented with both Se and vitamin C ($P<.025$). On the fifth week the uninfected chicks fed diets supplemented with either Se ($P<.025$) or vitamin C ($P<.005$) had higher feed intake than those fed the basal diet and those supplemented with both Se and vitamin C ($P<.025$). However, there was no significant difference between the chicks fed the basal diet and those fed diets supplemented with both Se and vitamin C.

Infection significantly reduced feed intake in all supplemented dietary groups one and three weeks after infection ($P<.005$) and in the group fed the basal diet one and two weeks postinfection ($P<.005$).

Within the infected group, the chicks supplemented with vitamin C or Se or both had higher intake than the chicks fed the basal diet ($P<.1$ - $P<.025$), on the fourth week.
On the fifth week the infected chicks on the basal diet had higher feed intake than those fed diets supplemented with Se ($P<.005$), with vitamin C ($P<.025$) or both ($P<.0005$). Also,

chicks fed either Se or vitamin C had higher intake than those fed both ($P<.05$).

Table (18) The Effect of Se, Vitamin C and E.coli Infection

Table (18) The Effect of Se, Vitamin C and E.coli Infection on Feed Intake of Chicks

Infection	Not Infected					Infected				
Time Wks / Diet	1	2	3	4	5	1	2*	3	4	5
Basal	13.10	27.24	47.77	68.68	61.97	15.48	25.85	28.06	45.73	68.63
Basal + Se	16.71	26.49	43.48	60.77	78.88	16.55	28.59	28.06	60.61	46.89
Basal + C	14.29	24.52	39.62	57.48	75.59	14.01	28.17	28.64	56.22	59.39
Basal + Se + C	15.04	30.62	42.81	55.37	64.12	14.09	27.48	27.99	59.31	34.59

SEM 4.64

Number of Birds in each group 15.

* The chicks were infected at two weeks of age.

Mortality Rate:

The addition of 0.3 ppm Se and 150 mg vitamin C to the basal diet significantly increased mortality ($P<0.025$) in both the infected and uninfected groups, Table (19). Infection resulted in a significantly higher mortality rate as compared to the uninfected group ($P<0.001$).

Table (19) The Effect of Se, Vitamin C and E.coli Infection on Mortality Rate of Chicks

Infection(1)	* Not Infected				Infected			
Diet (2)	Total # of Birds	Number Surviving	# Dead	% Mortality	Total # of Birds	Number Surviving	# Dead	% Mortality
Basal	12	11	1	8.33	13	8	5	38.46
Basal + Se	15	14	1	6.67	15	7	8	53.33
Basal + C	15	14	1	6.67	15	10	5	33.33
Basal + C + Se	12	8	4	33.33	14	6	8	57.14

(1) Infection significantly increased mortality ($P<.001$)

(2) Addition of .3 ppm Se and 150 mg vitamin C to the diet significantly increased mortality ($P<.025$) in both infected and noninfected groups.

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Antibody Titers:

One week after infection the chicks fed diets supplemented with either Se or vitamin C or both had significantly ($P<.05$ - $P<.0005$) higher antibody titers than those fed the basal diet, Table (20). There was no significant difference in antibody titers between chicks fed either vitamin C or Se.

During the second week postinfection, the addition of Se, vitamin C or both to the basal diet, significantly increased ($P<.1$ - $P<.0005$) antibody titers. Supplementation with vitamin C increased antibody titers more than supplementation with Se ($P<.0005$).

Three weeks postinfection, the addition of Se, vitamin C or both, still significantly ($P<.0005$) increased antibody titers, and the addition of both resulted in a further increase than either one alone, $P<.0005$). Also vitamin C resulted in higher antibody titers than Se ($P<.0005$).

Table (20) Log_2 Antibody Titers of Chicks Infected with E.coli and the Effect of Vitamin C and Se

Diet \ Time	Weeks After Infection 1	2	3
Basal	3.377 ± 0.346[a] (6)	4.44 ± 1.387[a] (7)	3.749 ± 0.249[a] (8)
Basal + Se	4.496 ± 1.327[b] (5)	4.833 ± 1.257[b] (7)	4.999 ± 1.00[b] (7)
Basal + C	4.25 ± 0.251[b] (5)	6.055 ± 1.293[c] (11)	6.498 ± 0.865[c] (10)
Basal + C + Se	3.827 ± 0.505[c] (6)	8.497 ± 0.999[d] (5)	8.055 ± 0.82 [d] (6)

Mean ± SD

( ) Number of Birds

abcd Different superscripts in the same column are significantly different (P<.1)

Experiment 4

This experiment was conducted to determine the effect of E.coli toxin on the chicks, and whether supplementation with vitamin E and Se will ameliorate the toxic effects. Several preliminary trials (1-5) were conducted to test the susceptibility of the chicks to E.coli toxin. Toxins from different sources and extracted by different methods were used.

Table (21) summarizes the results obtained in these trials.

Table (21) Effect of Three Different E.coli Endotoxins on Chicks:

	Breed and No. of Birds	Age	Endotoxin	Source	Effect
1	White Leghorn (6)	15-21d	350-400mg/Kg I/P	Difo lab 055:B5 phenol water extraction	3 birds died with s/c hemorrhage at site of injection - 3 were depressed for 1-2 days and were normal on recovery
2	Broiler type (2)	15d	266 mg/Kg I/V	Dr. Wilson PSU.0155:B5 phenol H_2O extraction	the 2 birds died in less than 24 hours showing signs of septicemia congestion of liver, intestines, kidney
3	White Leghorn (4)	15d	1 ml extract I/V	salt extract toxin from 078:T_3 Dr. Saif Ohio	All birds died .2 in 24 hours .1 on the 4th day and 1 by first week showing dehydration, cynosis, congestion of b.vs, liver, spleen, entritis hem. in peyers patches and tonsils and proventriculus
4	White Leghorn (4)	15d	1 ml extract I/P	salt extract toxin 078:T_3	1 died in 5-6 hours showing peticheal hemorrhage in liver congestion of spleen.
5	White Leghorn (4)	21d	0.5 ml extract I/V	salt extract toxin 078:T_3	1 died in 24 hours and 2 within 1 week (lesions -3-)

For this experiment E.coli toxin, extracted by 9% Nacl, was used. The 108 day-old chicks were divided into 6 groups in a 2 x 3 factorial experiment with a split plot design. They were fed the same corn-soybean meal basal diet with 10% cod liver oil described in experiment 1 and 2 without the addition of vitamin E and Se or supplemented with 0.2 ppm Se and 300 IU vitamin E/kg diet, (Table, 1).

At two weeks of age one third of the chicks fed each diet were injected in the brachial vein with 0.5 ml E.coli extract, one third were injected in the posterior thoracic air sac with 0.15 ml E.coli suspension, same as experiment 2, and one third were uninfected. Twelve extra chicks were fed each of the diets and one chick from each group was randomly chosen and injected I/V with purified endotoxin 0155.B_5 (Dr. Wilson), to determine whether chicks are susceptible to purified endotoxins. Due to the limited amount of endotoxin only two chicks were used.

General:

After being fed the deficient diet for two weeks, some chicks developed clinical signs of exudative diathesis, nutritional encephalomalacia (Figure 4) and died within a week. The chicks fed the deficient diet also had lower plasma Se and alpha tocopherol values than those fed the supplemented diet, at the end of the experiment, Table (22). The chicks injected I/V with 0.5 ml of the extract were depressed, anorexic, and unthrifty.

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The chicks that were injected I/V with 266 mg/kg of purified endotoxin provided by Dr. Wilson exhibited depressed growth immediately after injection, and died in less than 24 hours.

The injection of 078:T_3 strain of E.coli in the posterior thoracic air sac did not result in very high mortality as it did in experiment 1.

Table (22) The Effect Of Diet , E.coli Infection and E.coli Toxin on Plasma Tocopherol and Se Values.

Table (22) The Effect Of Diet , E.coli Infection and E.coli Toxin on Plasma Tocopherol and Se Values

Infection / Diet	Not Infected		Infected		Given Toxin	
	alpha tocopherol ug/ml plasma	Se ug/ml plasma	alpha tocopherol ug/ml plasma	Se ug/ml plasma	alpha tocopherol ug/ml plasma	Se ug/ml plasma
+E/Se	6.1 ± 1.99 (3)	.192 ± .05 (6)	5.163 ± .99 (4)	.217 ± .047 (6)	2.67 ± .158 (4)	.214 ± .041 (3)
-E/Se	T (4)	.041 ± .008 (4)	T (4)	.044 ± .007 (5)	T (4)	.036 ± .013 (3)

Mean ± Std. dev.
( ) number of birds
T only traces of tocopherol.

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Figure 4. Vitamin E-Se deficiency in chick.
Above : Nutritional encephalomalacia in vitamin E-Se deficient chick.
Lower left : Control chick fed diet supplemented with 300 IU vitamin E and 0.2 ppm Se.
Lower right : Exudative diathesis in vitamin E/Se deficient chick.

Weight gains:

Supplementation of the diet with 0.2 ppm Se and 300 IU vitamin E significantly increased the weight gain ($P<.1$), Table (23). Injection of the toxin or the E.coli microorganism significantly reduced the weight gains one week postinjection, in chicks fed both diets. The deficient chicks had lower weight gains than the supplemented group. Two and three weeks postinfection the survivors of the E.coli infection fed the deficient diet had no significant difference in weight gains from controls. Both the controls and those infected with E.coli had significantly higher weight gain than those given toxin ($P<.1$).

On the supplemented diet the control group still gained more weight than the group injected with toxin, two and three weeks postinjection; and the infected group three weeks postinfection. The infected chicks gained more weight than the chicks injected with toxin.

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Table (23) Weight Gain in gm/bird/day

The Effect of Vitamin E/Se, E.coli Infection and E.coli Toxin Injection on Weight Gain:

Time (weeks)		1	2*	3	4	5
Diet	Infection					
-E/Se	-	19.06	24.90	33.27	33.10	47.57
	toxin	18.88	28.86	18.30	20.27	34.59
	micro-organism	18.37	27.32	23.81	39.71	44.87
+E/Se	-	19.25	33.39	48.74	54.18	56.47
	toxin	16.85	32.22	21.93	30.45	27.95
	micro-organism	17.89	32.73	42.37	49.44	44.27

SE of mean 3.71
Number of chicks per group 18.
* Chicks were infected at the end of two weeks.

Feed Intake:

Supplementation of the diet with 0.2 ppm Se and 300 IU vitamin E significantly increased feed intake in the control group, at two weeks of age and in all the other groups at three, four, and five weeks of age.

Uninfected chicks fed the deficient diet, had significantly ($P<0.1$) higher feed intake than the chicks given the toxin, one, two and three weeks postinjection; and no significant difference in feed intake when compared to those of the infected group.

On the supplemented diet the uninfected control group had significantly ($P<0.1$) higher intake than the chicks

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given the toxin, one, two, and three weeks postinjection and the infected group one and two weeks postinjection.

The infected group had significantly higher intake than the chicks injected with the toxin, one, two and three week postinjection, (Table 24).

Table(24) Feed Intake gm/bird/day

The Effect of Vitamin E/Se, E.coli Infection and E.coli Toxin Injection on Feed Intake

Time (weeks)		1	2*	3	4	5
Diet	Infection					
-E/Se	-	20.80	36.22	53.08	62.43	90.10
	toxin	20.12	39.91	28.46	46.74	68.33
	micro-organism	21.65	36.64	44.26	71.68	90.25
+E/Se	-	21.48	45.74	78.07	99.76	111.79
	toxin	19.63	44.06	52.94	65.81	42.93
	micro-organism	20.27	39.64	65.64	89.05	105.07

SE of mean 5.517
Number of chicks per group 18.
* Chicks were infected at the end of two weeks.

Mortality Rate:

Mortality due to injection of E.coli microorganisms was significantly ($P<.001$) lower than mortality resulting from E.coli toxin, in both the deficient and supplemented groups. Supplementation of the diet with 0.2 ppm Se and 300 IU vitamin E significantly ($P<.001$) reduced mortality in the noninfected, infected and in chicks injected with toxin (Table 25).

Table (25) Effect of E/Se and Infection with E.coli or E.coli Toxin on Mortality Rate of Chicks

Infection(1)	* Not Infected				Toxin				E.coli			
Diet (2)	Total # of Birds	Number Surviving	# Dead	% Mortality	Total # of Birds	Number Surviving	# Dead	% Mortality	Total # of Birds	Number Surviving	# Dead	% Mortality
-E/Se	51	39	12	23.53	18	5	13	72.22	18	11	7	38.88
+E/Se	54	53	1	1.87	18	15	3	16.66	18	17	1	5.55

* Data pooled from two experiments.

(1) Toxin injection resulted in significantly higher mortality rate than E.coli infection ($P<.001$)

(2) Supplementation of the diet with 0.2 ppm Se and 300 IU vitamin E significantly reduced mortality in all groups ($P<.001$).

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Gross Lesions and Histopathological Examination:

The uninfected deficient chicks had greenish gelatinous exudate under the skin especially in the head and neck region (Figure 5) and hemorrhages on the cerebellum. Histopathological examination revealed myopathy of the skeletal muscle characterized by hyalinization and edema also disintegration and degeneration in muscle fibers (Figure 6). The brain had vacuolation in the cerebellum and degeneration of the granular layer of the white matter together with congestion, and the neurons had pyknosis, as seen in experiment 1.

On gross examination, chicks that died from E.coli toxin were dehydrated, had cyanosis, and vasocongestion. Livers, muscles, lungs and spleens were congested and the kidneys had subcapsular hemorrhage. The intestines had hemorrhagic spots on the Peyers patches and cecal tonsils. The proventriculus also had hemorrhages at the junction with esophagus. (Figure 7)

On microscopical examination there were focal areas of necrosis in the liver with some areas of hemorrhage (Figure 8). The kidneys had necrosis and vacuolation especially in the proximal convoluted tubule and medulla (Figure 9). The spleen was congested as were the intestines.

Chicks that were injected I/V with 266 mg/kg purified endotoxin had lesions of subcutaneous hemorrhage and

gelatinous exudate. They had congestion and hemorrhage in the intestines, Peyers patches, cecal tonsils and adipose tissue. Also there was congestion in the lungs, liver, spleen and muscles, together with subcapsular hemorrhage in the kidneys.

On histopathological examination there was congestion and edema in the skin and muscles. The heart was congested with its fibers separated. Liver and spleen were also congested. The intestines were very congested wsith considerable desquamation necrosis and pycnotic nuclei. The lungs and air sacs had edema, were congested and had some serofibrinous exudate in the lumen of the lung. The kidneys were also congested, had separated cells with pyknotic nuclei especially in the proximal tubules.

The chicks that died due to E.coli infection had subcutaneous hemorrhages, congestion of liver, kidney, intestines and lungs. Some chicks also developed pericarditis, peritonitis and airsacculitis, as seen in experiment 1.

Microscopically the lungs had necrotizing broncho-peribronchial and intestinal pneumonia which was focally severe. The heart had superficial pericarditis and epicarditis. The spleen had lymphoid necrosis and so did the bursa. Bacteria had accumulated in the lamina propia of proventriculus, intestines and spleen.

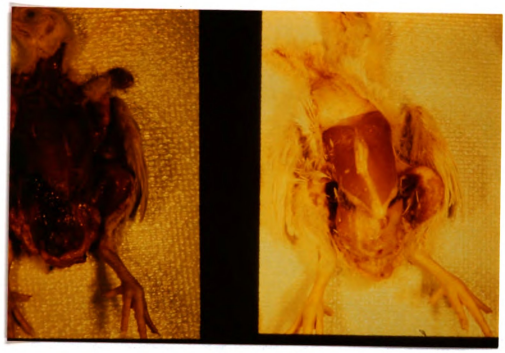

Figure 5. Left : Exudative diathesis in vitamin E/Se deficient chick.
Right : Control chick fed diet supplemented with 300 IU vitamin E and 0.2 ppm Se.

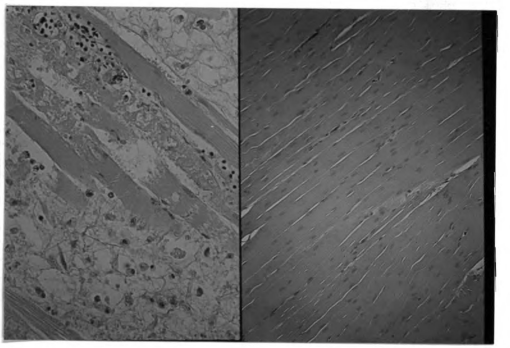

Figure 6. Microscopic appearance of skeletal muscle. Hematoxylin and eosin.
Left : Muscular dystrophy in vitamin E/Se deficient chick. Myopathy, disintegration, edema and cellular infiltration.(25X)
Right : Control chick fed diet supplemented with 300 IU vitamin E and 0.2 ppm Se.(10X)

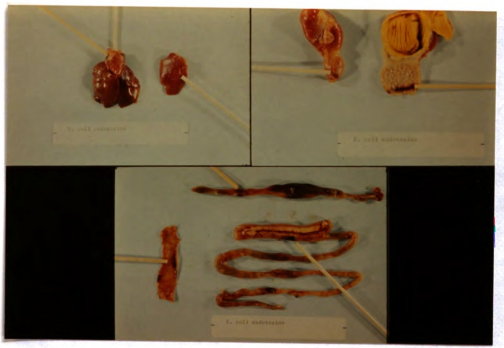

Figure 7. Lesions of E.coli toxin in chicks.
Upper left : Congestion of liver.
Upper right : Hemorrhage in proventriculus.
Below : Hemorrhages in Peyers patches and cecal tonsils.

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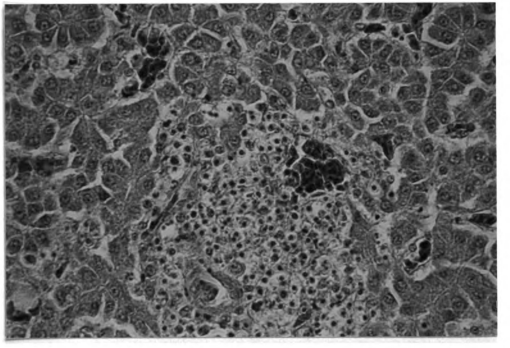

Figure 8 : Microscopic appearance of liver of a chick injected with E.coli toxin. Congestion and foccal necrosis. Hematoxylin and eosin.(25X)

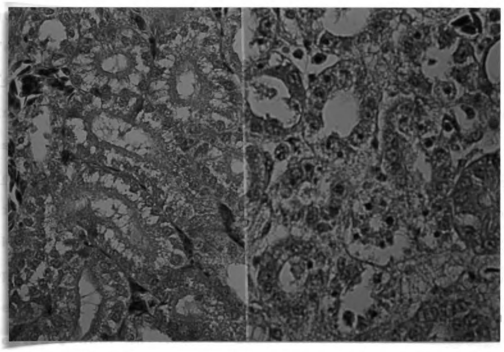

Figure 9. Microscopic appearance of kidney of chick injected with E.coli toxin.
Left : Congestion and vacuolation in proximal tubule.(40X)
Right : Necrosis in medulla.(40X). Hematoxylin and eosin.

Bacteriological examination:

E.coli was isolated from all of the livers, ascitical and pericardial fluids of all dead chicks. No other bacteria was present.

Antibody Titers:

Effect of diets: Addition of 0.2 ppm Se and 300 IU vitamin E to the basal diet significantly ($P<.005$) increased antibody titers three weeks postinjection, in the chicks injected with the toxin, and two weeks postinjection in the infected group ($P<0.1$), Table (26).

Effect of the infection: The chicks given the toxin had significantly ($P<0.005$) higher antibody titers than infected ones.

Effect of time: In the deficient group given the toxin, antibody titers increased significantly in the first two weeks and then significantly decreased ($P<.0005$). In the supplemented group antibody titers increased one, two and three weeks postinjection ($P<.025$ - $P<.0005$). In the infected group, antibody titers significantly decreased two weeks after infection in the deficient group ($P<.005$), and significantly increased two weeks after infection in the supplemented group ($P<.025$).

Table (26) Effect of Diet, E.coli Infection and Toxin Injection on Humoral

Immune response of chicks (log_2 titers)

Infection	E.coli toxin			E.coli microorganism		
Wks. post infection diet	1	2	3	1	2	3
-E/Se	6.322(5) ± 2.51	8.16(4) ± 1.87	5.322(4)[a] ± 0.586	3.322 (11) ± 0.575	2.104(6)[a] ± 0.19	1.993(11) ± 0
+E/Se	6.77(11) ± 1.347	7.848(11) ± 1.463	9.606(8)[b] ± 1.672	3.268(11) ± 0.91	3.158(11)[c] ± 0.765	2.242(11) ± 0.249

Means ± Std. Dev.
( ) # of Birds
ab significant at P<.0005
ac significant at P<.01

Experiment 5

In this experiment, the effect of vitamin E and Se deficiency and supplementation on chicks vaccinated with ND vaccine (a live attenuated viral vaccine) was determined. 135 chicks were used in a 2 x 2 factorial experiment with a split plot design.

The chicks were divided into 4 groups with 3 replicates per group with 11 birds in each (12 in one replicate). The diets fed were as mentioned in experiment 1, 2, 4. At two weeks of age one group of chicks fed each of the two diets was administered I/M in the left thigh with 0.5 ml ND vaccine.

General:

Chicks fed the deficient diet started developing clinical signs of E/Se deficiency in two weeks. Signs of nutritional encephalomalacia and exudative diathesis were as described in experiment 4.

The deficient chicks had also lower plasma Se and alpha tocopherol values than the supplemented groups at the end of the experiment, (Table 27).

Table (27) Se and alpha tocopherol plasma values in chicks fed basal or E/Se supplemented diets.

Diet	Se ug/ml plasma	alpha tocopherol ug/ml plasma
- E/Se	.018 ± .0028 (8)	T (6)
+ E/Se	.103 ± .008 (8)	2.48 ± .83(3)

Mean ± Std. Dev.
( ) # of samples analyzed
T Trace

Weight gains:

Given in Table (28) are the results of weight gain. Supplementing the diet with 0.2 ppm Se and 300 IU vitamin E/kg diet significantly increased the weight gain of chicks at two weeks ($P<.25$) and three weeks ($P<.0005$). Vaccination significantly ($P<.25$) reduced weight gain in chicks fed the deficient diet, but not in chicks fed the supplemented diet.

Table (28) Effect of E/se and ND Vaccine on Weight Gain of Chicks (gm/bird/day)

Time / Weeks	Not vaccinated			Vaccinated		
Diet	1	2	3	1	2*	3
-E/Se	13.28	22.42	26.00	13.06	23.13	21.00
+E/Se	14.00x	28.71x	43.71x	13.17	28.75	41.57

SEM = 1.654
Number birds in each group 33
x Number of birds 36
* The chicks were vaccinated at two weeks of age.

Feed Intake:

Supplementing the diet with 0.2ppm Se and 300 IU vitamin E significantly increased feed intake during the second ($P<.05$) and third week ($P<.0005$).

Vaccination caused a nonsignificant decrease in feed intake in chicks fed both diets, (Table 29).

Table (29) Effect of E/Se and ND Vaccine on Feed Intake

(gm/bird/day)

Time Weeks	Not vaccinated			Vaccinated		
Diet	1	2	3	1	2*	3
-E/Se	14.97	31.67	41.00	14.93	32.33	38.67
+E/Se	15.23x	39.20x	68.00x	14.93	38.33	64.00

SEM = 2.239
Number of birds in each group 33.
x Number of birds 36
* The chicks were vaccinated at two weeks of age.

Mortality Rate:

Chicks fed diets deficient in vitamin E and Se had significantly ($P<.001$) higher mortality rate than those fed diets supplemented with 300 I.U. vitamin E and 0.2 ppm Se. Injecting the chicks with 0.5 ml ND vaccine increased the number of chicks that died in the deficient group, but not significantly. While in the supplemented group vaccination did not result in any change in mortality rate, (Table 30).

Gross Lesions and Histopathological Examination:

Lesions and histopathology of nutritional encephalomalacia, muscular dystrophy and exudative diathesis, due to E/Se deficiency, were as in experiment 4.

Table (30) Effect of Vitamin E/Se and Newcastle Disease Vaccine on Mortality in chicks

diets	vaccination	total # of birds	# surviving	# dead	% mortality
-E/Se	-	33	24	9	27.27
	+	33	20	13	39.39
+E/Se	-	36	36	0	0.00
	+	33	33	0	0.00

DISCUSSION

Experiments 1 and 2

The chicks fed the commercial type basal diet unsupplemented with vitamin E developed clinical signs and lesions of nutritional encephalomalacia as summarized by (Ames, 1956) and produced earlier using a purified vitamin E deficient diet (Dam, 1944). The deficient chicks also had lower plasma alpha tocopherol values (.063 ± ug/ml plasma) as compared to the vitamin E supplemented chicks (1.76 ± .42 ug/ml plasma); vitamin C seems to have a sparing effect on vitamin E, since the addition of vitamin C to the diet, increased plasma alpha tocopherol values (.225 ± .14 and 2.76 ± .7 ug/ml plasma) for the vitamin E deficient and vitamin E supplemented diets, respectively.

The chicks infected with E.coli developed lesions of airsacculitis, fibrinous pericarditis and perihepatitis, which are similar to the pathological changes described for an E.coli infection (Gross, 1957).

Weight gains:

In experiment 1, supplementing the diet with vitamin E, C or both significantly increased weight gain before infection; while in experiment 2, there was no significant difference in weight gain between the four dietary groups. These results confirm the controversy about the role of vitamin E regarding its effect on weight gains. These inconsistant results agree with Colango et al. (1984) who

reported an increase in weight gain of nonimmunized chickens infected with E.tenella and fed vitamin E, while Julseth (1974) reported no significant improvement in body weight gains in turkeys when fed diets supplemented with vitamin E.

In this research infection reduced weight gain significantly in all dietary groups when compared to the uninfected controls, except in the group fed both vitamins, thus indicating that the addition of both vitamins E and C helped in overcoming the weight loss due to infection. The addition of either vitamin E or C restored weight gain one week postinfection, while the infected chicks fed the deficient diet had lower weight gains than the uninfected controls two and three weeks postinfection. These results were similar to the research reported by Heinzerling et al. (1974) in that chicks infected with E.coli gained less weight than uninfected controls, and that dietary vitamin E restored weight gain of the infected to that of the uninfected chicks.

Feed Intake:

In experiment 1 supplementing the diet with both vitamins increased feed intake as compared to that of chicks fed the basal diet. This may in part explain the improved weight gain mentioned previously. In experiment 2 feed intake was not significantly affected by diets, except at three and five weeks where the chicks fed diets supplemented with vitamin C had higher feed intake. Infection reduced

feed intake in all groups. The chicks fed diets supplemented with vitamin E or both vitamins were back to normal feed intake within two weeks, while the chicks fed the basal diet continued to have lower feed intake than the uninfected controls. Within the infected group, chicks fed both vitamins had higher feed intake than those fed the basal diet and those fed vitamin E supplemented diet.

Mortality:

The addition of vitamin E to the basal diet significantly reduced the mortality rate in both experiments 1 and 2. Vitamin C reduced the mortality rate due to vitamin E deficiency. Dam et al. (1948) reported that the addition of 0.5% ascorbic acid to the diet delayed and minimized the tendency for exudation due to vitamin E deficiency. The diets they used were not supplemented with Se, thus the chicks developed exudative diathesis. The effects on nutritional encephalomalacia were not reported due to its low incidence in that experiment. Heinzerling et al. (1974a) reported an increased protection against a relatively moderate E.coli infection in chicks given 150 or 300 mg Dl. alpha tocopherol. Similar results were also reported by Julseth (1974) in turkeys. In this research infection resulted in high mortality as compared to uninfected controls and the diet did not appear to have any significant effect on mortality within the infected group, due to the high virulence of the E.coli strain (078:T_3)

which was reported to be of high pathogenicity (Rosenberger, 1985).

Immune Response:

Addition of vitamin E to the basal diet significantly increased the antibody titers as measured by hemagglutination one, two and three weeks postinfection. This is in agreement with Tengerdy et al. (1972) who reported an increase in the antibody titers as measured by hemagglutination or plaque forming cells in chicks fed vitamin E seven days postinfection. Similar results were also reported by Tengerdy et al., 1974a; Marsh et al., 1981; Heinzerling et al., 1974a; and Julseth, 1974.

Addition of vitamin C increased antibody titers only during the third week. Vitamin C has been reported to enhance the immune response by increasing cell-mediated cytotoxicity (Anthony et al., 1979 and Fraser et al. 1980) or by enhancing phagocytosis (Shilotri, 1977a and Miller, 1969). Addition of both vitamins significantly increased the antibody titers one and two weeks postinfection, suggesting a synergistic effect of both vitamins in enhancing the immune response.

The effect of both vitamins on reducing mortality and enhancing the immune response and the sparing effect of vitamin C on plasma alpha tocopherol support the results of Chen and Thacker (1975) who reported that vitamin C acts to spare the metabolism of vitamin E and partially reverse the

biochemical changes due to vitamin E deficiency. This may also be due to the fact that both vitamins act as antioxidants and that vitamin C spares vitamin E by regenerating the vitamin E radical resulting from the reaction of vitamin E with lipid peroxides, (Packer et al., 1979; Tappel, 1968; Bascetta et al., 1983; and Niki et al., 1984).

Experiment 3

The chicks on the basal diet not supplemented with Se did not develop any clinical signs of exudative diathesis. Guenter and Bragg (1977) reported that chicks fed diets deficient in Se (.017 ppm) and which contained 50 IU vitamin E/kg diet, developed exudative diathesis and that vitamin E only delayed the signs. They also reported that chicks fed diets containing .04 ppm Se developed no exudative diathesis, so the basal diet used in this experiment appears to be not completely deficient in Se. Se deficiency in the chicks has also been reported to cause pancreatic lesions (Thompson and Scott, 1970). The absence of any pancreatic lesions in the chicks in this experiment also indicates that the basal diet was not completely deficient in Se, since pancreatic atrophy is effectively prevented by .02 - .05 ppm Se in the presence of 10 - 15 IU vitamin E/kg diet (Combs Jr., 1981). In spite of not having any deficiency lesions, the chicks fed the deficient diets had lower plasma Se

values (.042 ug/ml) than those supplemented with Se (.131 ug/ml). Addition of vitamin C to the basal diet did not cause any change in plasma Se value (0.04 ug/ml), while the addition of vitamin C to the supplemented diet slightly increased plasma Se (0.149 ug/ml).

Lesions and histopathological changes following E.coli infection were similar to experiments 1 and 2.

Weight Gains and Feed Intake:

The results of the weight gains and feed intake of the chicks in this experiment were inconsistent except for the fact that the infection reduced weight gain and feed intake as compared to the uninfected controls.

Generally, the chicks fed the basal diet had higher weight gains and similar intakes compared to the other dietary groups. The chicks fed the basal diet supplemented with 0.3 ppm Se and 150 mg vitamin C had the lowest weight gains and feed intake.

Mortality:

As with weight gain and feed intake the mortality rate was highest in the group fed the diet supplemented with 0.3 ppm Se and 150 mg vitamin C. In the uninfected group addition of either Se or vitamin C reduced the mortality rate as compared to the basal diet supplemented with both Se and vitamin C.

Infection significantly increased mortality rate as compared to uninfected controls in all dietary groups, especially

when both Se and vitamin C were fed together.

Apparently from these results and from the absence of Se deficiency lesions in the chicks fed the basal diet, the basal diet was not completely deficient in Se especially with the addition of 50 IU vitamin E/kg diet and ethoxyquin. This is supported by the findings of Thompson and Scott (1968), who mentioned that the Se requirement was considerably lower in chicks receiving high levels of vitamin E, and that it could be met by Se present in all ingredients but the most highly purified diets. Also, the addition of ascorbic acid may have reduced the Se requirements by increasing the biologic utilization of dietary Se due to its action as an antioxidant (Combs and Scott, 1974). Ascorbic acid also increases the activities of Se containing enzyme GHS.Px in plasma and increases enteric absorption of Se which is accompanied by an apparent reduction in dietary Se requirement, (Combs Jr. and Pesti, 1976).

So the depression in weight gain and feed intake and the higher mortality in the group fed both Se and vitamin C, indicates that in the presence of high vitamin E, an antioxidant and vitamin C, the addition of as little as .3 ppm Se might have an adverse effect on the chicks.

Immune Response:

Supplementation of the diet with 0.3 ppm Se significantly increased antibody titers as compared to

titers in chicks fed the basal diet. This confirms the results of Spallholz et al., (1973a, 1973b and 1975) who reported an increase in the immune response of mice fed 1 to 3 ppm Se. Vitamin C also increased antibody titers and the addition of both Se and vitamin C resulted in a further increase as compared to the basal diet two weeks postinfection, suggesting a synergistic effect of Se and vitamin C on the immune system as contrasted to their effect on weight gain, feed intake and mortality.

Experiment 4

The chicks injected with Difco endotoxin manifested resistance to large amounts of endotoxins. This confirms the results reported earlier by Ball et al., 1962; Jordan and Hinshaw, 1964; Cole and Boyd, 1965 and Alder and DaMassa, 1978, for the resistance of chickens to purified endotoxins. Conversely, injecting two chicks with 266 mg/kg purified endotoxin provided by Dr. Wilson caused death of the two injected chicks in less than 24 hours. These chicks had severe signs of septicemia and lesions in the kidneys, liver, heart and intestines. This indicates the chicks may not be totally resistant to purified endotoxins but more work needs to be done using a dose-response curve to evaluate the effect of this purified endotoxin on the chicks as compared to E.coli infection. The E.coli strain

apparently plays an important role in toxicity, since the production of toxins depend on many factors concerning growth conditions, including media, incubation time and temperature, (Truscott, 1973), thus, the procedures developed for a particular strain may be less than ideal for other strains.

Truscott (1966) produced necrotic liver lesions and some mortality in two week old chicks when injected with 1 ml of E.coli toxin extracted by NaCl. We used 9% NaCl to extract toxin from 078:T_3 strain of E.coli and injected 0.5 ml I/V in chicks at two weeks. The chicks developed severe lesions in the kidneys, livers and intestines, and some developed lesions of pericarditis and perihepatitis, which are the classical lesions of E.coli infection. This suggests an active role of E.coli toxin in E.coli infection, and has not been investigated extensively in previous research.

The lesions caused by NaCl extracted E.coli toxin may be due to other wall components besides lipopolysacchride which are removed by salt extraction and result in toxicity, (Alder and DaMassa, 1978).

The chicks fed the basal diet unsupplemented with vitamin E and Se had clinical signs and lesions of exudative diathesis, nutritional encephalomalacia; and muscular dystrophy as described for vitamin E and Se deficiency (Scott, 1982). The chicks also has lower plasma Se (.04

ug/ml) and alpha tocopherol (traces) when compared with the values in chicks supplemented with vitamin E and Se (.2 ug Se/ml and 4.66 ug alpha tocopherol/ml). The chicks infected with E.coli had lesions described for E.coli infection in experiment 1, 2 and 3, and the chicks injected with E.coli toxin had similar lesions.

Weight Gains:

Supplementing the basal diet with 300 IU vitamin E and .2 ppm Se increased weight gain in the noninfected group, and in the infected group and that given the toxin one and two weeks postinjection. Vitamin E and Se had been reported to increase weight gain in chicks infected with Eimeria (Jensen and Johnson, 1978; Colango et al, 1984b; and Colango et al. 1983). Infection reduced weight gain. The chicks injected with E.coli toxin had the lowest weight gains.

Feed Intake:

Supplementing the diet with 300 IU vitamin E and .2ppm Se increased feed intake in the infected and uninfected groups. Infection reduced feed intake as compared to uninfected controls. The chicks injected with the toxin had the lowest feed intake and, therefore, the lowest weight gains.

Mortality:

Supplementing the diet with 300 IU vitamin E/kg and .2 ppm Se significantly reduced mortality in the noninfected as well as in the infected group and those injected with the toxin. Similar results were reported for the effect of E/Se in reducing mortality in chickens infected with E.tenella (Jensen and Johnson, 1978 and Colango et al., 1984b) and also in chickens with pale bird syndrome (Colango et al., 1983). The chicks injected with E.coli toxin had higher mortality than those infected with E.coli.

Immune Response:

The addition of 300 IU vitamin E and .2 ppm Se to the basal diet significantly increased the antibody titers. The results are in agreement with those of Marsh et al., (1981) who reported an increase in the humoral immunity of chickens supplemented with vitamin E and/or Se. The chicks injected with the E.coli toxin had higher antibody titers than those infected with E.coli.

Experiment 5

The chicks fed the deficient diet developed clinical signs and lesions of vitamin E/Se deficiency mentioned previously. They also had lower plasma Se (.018 ug) and alpha tocopherol (traces) as compared to chicks fed the supplemented diet (.103 ug Se/ml and 2.48 ug alpha tocopherol/ml).

Weight Gains:

Addition of 300 IU vitamin E/kg and .2 ppm Se to the basal diet increased weight gains as has been reported in Experiment 4. Vaccination reduced weight gain in chicks fed the deficient diet but not in chicks fed the supplemented diet, indicating that vitamin E and Se reduced the depression of weight gain due to vaccination.

Feed Intake:

Supplementing the diet with 300 IU vitamin E and .2 ppm Se significantly increased feed intake and weight gain as reported in Experiment 4. Vaccination did not cause any significant change in feed intake.

Mortality:

Addition of 300 IU vitamin E and .2 ppm Se to the basal diet reduced mortality rate as in Experiment 4. Vaccination numerically increased mortality in the deficient group but not in the supplemented group, indicating a protective role of vitamin E and Se against post-vaccination reaction.

Immune Response:

In this experiment the antibody titers could not be determined after vaccination since the chicks had positive antibody titers, as measured by hemagglutination inhibition, before vaccination indicating the presence of maternal antibodies.

SUMMARY AND CONCLUSIONS

Five experiments, using 120 chicks in each, were conducted to determine the effect of vitamin E, C and Se on the performance and immune response of chicks infected with E.coli or vaccinated with Newcastle disease vaccine. In addition, the susceptibility of chicks to E.coli toxin and the comparative lesions to E.coli infection were evaluated.

The chicks were fed either a basal diet or the basal diet supplemented with nutrients investigated. The chicks fed the vitamin E-deficient diet and those fed the vitamin E/Se-deficient diet had lesions of nutritional encephalomalacia, exudative diathesis and muscular dystrophy. The chicks infected with E.coli had lesions of pericarditis, perihepatitis and airsacculitis.

The results of this research provide the basis for the following conclusions:

1. Supplementing the diet with 300 IU vitamin E/kg reduced the mortality rate due to E-deficiency, had inconsistent influence on weight gain and feed intake. It increased the ability of chicks infected with E.coli to resist disease, restored the weight loss due to infection and increased the hemagglutination titers against the E.coli lipopolysaccharide antigen.

2. Addition of vitamin C reduced mortality due to vitamin E-deficiency. Addition of both vitamins increased disease resistance, restored weight loss due to infection and increased antibody titers.
3. Supplementing the diet with 0.3 ppm Se in the presence of 50 IU vitamin C increased mortality rate and lowered weight gain and feed intake as compared to the unsupplemented group. However, the addition of either Se, vitamin C or both increased antibody titers against E.coli lipopolysaccharide antigen.
4. Mortality rate due to infection was high due to the high virulence of the E.coli strain used, and consequently masked any dietary effect.
5. Chicks were resistant to purified endotoxin purchased from Difco, while the chicks injected with purified endotoxin prepared from another strain of E.coli died in less than 24 hours, indicating that the strain of E.coli used rather than the method of extraction influences its toxicity.
6. Injecting the chicks with E.coli toxin extracted with 9% NaCl resulted in high mortality rate and caused lesions of pericarditis and airsacculitis similar to E.coli lesions suggesting an active role of the toxin in E.coli infection. The

mortality rate was higher and the weight gains and feed intake lower in the chicks injected with toxin than those infected with E.coli.

7. Supplementing the diet with 300 IU vitamin E and 0.2 ppm Se reduced mortality rate, increased weight gain and feed intake in infected, noninfected and the chicks given the toxin as compared to the deficient group.

8. The chicks fed vitamin E/Se-deficient diet and vaccinated with Newcastle disease vaccine had lower weight gain than the supplemented group, suggesting that the addition of 300 IU vitamin E and 0.2 ppm Se to the chicks' diet before vaccination reduced the stress of vaccination reaction.

APPENDIX "A"

BACTERIOLOGY

1. Bacterial Count: (Benson, 1977)

   a. Serial dilutions of 18-20 hours broth cultures in phosphate buffered saline - PBS (Delbacco's PBS) were prepared using a sterile pipette for each dilution.

   b. From each dilution 10^{-6}-10^{-12} 0.1 ml of the diluted culture was transferred in 3 empty Petri dishes.

   c. Nutrient agar, cooled to 50°C was then poured into each plate. When the media solidified, the plates were incubated, inverted, at 37°C for 24 hours.

   d. From these dilution plates those with 30-300 colonies were selected.

   e. The number of colonies were counted using Darkfield Quebec Colony Counter (American Optical Cooperation).

   f. The average number of colonies for each dilution was determined.

   g. The number of microorganisms per ml of culture was calculated by multiplying the number of colonies counted by the dilution factor.

2. Gram Staining Procedure (Benson, 1977)

   a. Smear is covered with crystal violet. Let stand for 20 secondsse white fumes of $HClO_4$ remove fuming flasks.

   b. Briefly wash off stain with distilled water.

   c. Cover smear with Gram's iodine for 60 seconds.

   d. Pour off Gram'd iodine and flood the smear with 95% ethyl alcohol for 20 seconds.

   e. Rinse slide with distilled water.

   f. Cover with safranin for 20 seconds.

   g. Wash with distilled water, blot with bibulous paper and let dry at room temperature.

   h. Examine under oil immersion lens.

3. Stains:

   a. Crystal Violet Stain. solution A: Dissolve 2.0 gm of crystal violet (85% dye content) in 20 ml of 95% ethyl alcohol. solution B: dissolve 0.8 gm ammonium oxalate in 80.0 ml distilled water. Mix solutions A and B.

   b. Gram's Iodine (Lugol's)

      Dissolve 2.0 gm of potassium iodide in 300 ml of distilled water and then add 1.0 gm iodine crystals.

   c. Safranin

      Safranin O (2.5% sol'n in 95% ethyl alcohol) - 10.0 ml; Distilled water 100.0 ml

APPENDIX "B"

LABORATORY ANALYSIS

1. Selenium: Whetter and Ullrey (1978)

   A. Reagents:

      1. 2-3 Diamino-napthalene = DAN
      2. Disodium dihydrogen ethylene diaminetetra acetate dihydrate = EDTA - acid form 14.2 g/L
      3. Cresol red .01 g in 1 ml H_2O + 1 drop 1:1 NH_4OH. Make up to 50 ml.
      4. Cyclohexane = chex
      5. Baker conc. HCL 1:9 and 1:99.

   B. Procedure:

      1. Preparation:
         a. Place samples in duplicates in 2 50 ml E flasks. Add 1 bead.
         b. Add 2 ml conc. HNO_3 and 2 ml $HClO_4$.
         c. Make duplicate blanks and .1, .2 and .3 Mg/ml standards.
      2. Digestion:

         To complete oxidize all Se and to drive out HNO_3.

         a. Hot plate to #2.4. Surround the lower part of the flasks with a double strip of foil.
         b. When the first flasks show the dense white fumes of $HClO_4$ remove fuming flasks.

c. Put the cooled flasks back on. When all flasks are fuming and bead bouncing, turn hot plate to Low. Set timer for 10 minutes.

d. Monitor the digestion. You should see a reflux action.

e. At the end of the digestion period, the $HCLO_4$ should be clear and colorless. Remove finished samples to cool.

f. Add 2.0 ml 1:9 HCl and place them all back on the hot plate at low. When the flasks are refluxing, set the timer for 15 minutes.

g. Remove flasks to cool in the hood.

3. Neutralizing, chelating and complexing:

a. Repipet 4.5 ml EDTA twice into each flask.

b. Repipet 1 ml conc. NH_4OH into each. Swirl. Add 2 drops of cresol red solution.

c. Add drops of conc. NH_4OH to orange pink.

d. Add 5 ml DAN, swirl and place on hot plate at low for 10 minutes.

e. Set flasks to cool. Now you have Se.DAN solution.

4. Extraction, clearing and reading:

a. Repipet 5.6 ml cyclohexane into each mixture.

b. Place flasks into the shaker. Gradually increase speed to 5.6. Set the timer for 5 minutes.

c. Fill flasks to neck area with DDH_2O.

d. With a Dispo pipet and small bulb, transfer the cyclohexane dissolved Se.DAN (top layer) to a tube.

5. Reading:

Read samples on Spectrofluorometer. Excitation at 367 m and emission at 518.

6. Calculation:

Do a curvilinear regression (concentration is y and RFE is x)

2. Alpha tocopherol (Bieri et al., 1979)

a. Materials:

The solvents are methanol, ethanol, hexane and water, all HPLC grade. We use Eastman Kodak 6340 d-alpha tocopherol, Eastman 6679 d- -tocopheryl acetate, Eastman 4640 Ascorbic acid and Sigma B-1378 butylated hydroxy toluens (BHT). Nitrogen is also needed.

b. Standards:

D-alpha tocopherol stock solution. Warm the viscous oil to room temperture. Tare a clean bottle/vial.

Open the alpha tocopherol and dip a clean glass rod into the oil. Wipe off the oil on the inside of the bottle/vial and weigh. Dissolve in sufficient ethanol to make about 2 mg/ml. Protect from light, layer with N_2 and freeze.

D-alpha tocopheryl acetate stock solution. See above d-alpha tocopherol.

D-alpha tocopherol stock solution. This may be used as an internal standard if d-alpha tocopheryl acetate is to be measured. Make up to about 4 mg/ml with ethanol. Calibration of standards, according to NBS. Daily.

D-alpha tocopherol working solution. Dilute the stock 2 mg/ml 1:19 in ethanol. Set the B-G spec at 292 nm, UV, slide out, off-set on. Pipet 0.5 mls of ethanol into two quartz cuvettes. Zero #1 with the slit adjustment, than #2 with knob 2. Add 0.5 mls of the working solution to each cuvette, mix with a dispo pipet and read the absorbance. Average the absorbance, multiply by 2 and divide by .00758 to calculate the actual concentration. The extinction coefficient of alpha tocopherol in ethanol is 75.8 (NBS). Dilute the working solution 1:9 for assay use.

D-alpha tocopheryl acetate working solution. Dilute the stock standard 0.50 + 9.50. Use this 100 ul of this 100 ug/ml. Does not need calibration.

Using the actual concentration of the working solution, calculate the volume to use to yield 1-10 ug/ml alpha tocopherol and 0.20-1.0 ug/ml retinol when

picked up in 1.0 ml mobil phase. This will bracket most samples.

c. Preparation

Select 24-40 15x150 mm pyrex tubes, checking that the caps are tight. Soak in HNO_3:H_2) 1:1 for 30 min, rinse %X with DDH_2O, 2X with HPLC ethanol and #X with HPLC hexane. Clean the 25 ml E flasks the same.

Make and filter 1L of mobil phase methanol: water (Ch_3OH:H_2)) 95:5. The solution is degassed by waters vacuum filtration unit. However, sonication for 10 min will ensure against bubbles. This mobil phase can be recycled. Do this just before using. This mobil phase is very prone to bubbles.

Make saturated ascorbic acid 34 g in 100 ml D DH_2O.

Set up repipet dispensers containing ethanol, hexane spiked with 0.05% BHT, and 100 ml of methanol.

d. Procedure:

Place the tubes in a rack, recipet 2 mls of ethanol into all tubes, add 0.3 mls sat. ascorbic acid and 0.100 of the 100 ug/ml -tocopheryl acetate. Pipet 1.0 ml of HPLC water into tubes 1-4 in place of the plasma sample. Leave a blank and make 3 alpha tocopherol standards, as previously described. N_2 and cap. Add 1 ml in duplicate of each of the samples to the remaining tubes. N_2 and cap after each pair.

Lock 20 tubes in the multi-vortexer and pulse 6X at top speed. Remove, uncap, and repipet 3 mls of BHT-hexane into all tubes. Layer with N_2, cap, and pulse-vortex at about 80 for 1 min. Transfer the tubes to the rack and cover with foil to carry to the centrifuge.

Centrifuge at 2500 rpm at 5°C for 10 min.

With a dispopipet transfer most of the top hexane layer to a 25 ml E flask. REpipet another 3 mls of BHT-hexane into the tube. Continue. Layer all tubes with N_2, cap and repeat vortex, centrifuge and transfer.

Place the combined extracts in a vacuum oven at room temperature. Pack the trap with ice and evaporate dry. Remove the flasks, cover with foil to carry to the N_2 and layer the oils with N_2. Repipet 1.0 mls of methanol into each flask. Lightly N_2 and cap.

Filter each extract into a 1/2 dram screw-cap vial using a glass syringe and disposable 0.45 um filter. The filter can b e used 10 times or more by rinsing between samples. Layer the vials with N_2 and cap.

While the extracts are drying, the tubes can be vortexed to loosen the pellet, brushed, rinsed with tap water, soaked in HNO_3:H_2O 1:1, rinsed with DDH_2O and set in the warm room to dry or used again for another batch. The flasks are cleaned by rinsing 3X with HPLC

hexane.

HPLC Set-up.

Insert the 280 filter and aperture in one channel, the 313 set in the other. Set the 280 channel at 0.01 sensitivity and the 313 channel at 0.05. Slowly increase pump speed to 2.5 ml/min $CH_3OH:H_2O$ 95:5. Allow to run until the baseline is steady. Turn on the recorder and set at 0.25 cm/min. Leave the other controls alone except for the zero. Check that the polarity is correct by turning the zero knob on the detector and watching the absorbance readout per pen shift. The pens should move towards the center with an increase in the readout.

Make duplicate 1:9 dilutions of the d- -tocopheryl acetate with methanol in a 1/2 dram vial, N_2 and cap.

Inject 100 ul of the Tac solution, continue with the standards and samples. Repeat the Tac injection half-way and again at the end.

If apocaretinal is used as an internalstandard use 436 filter and aperture in the other channel.

e. Calculations:

Measure the record the peak heights of the d- -tocopheryl acetate, and alpha tocopherol. Divide the average peak height of the unprocessed Tac (monitor or internal standard) by the extracted Tac peak of each standard or sample trace and multiply by the alpha

tocopherol peak height. Use the linear regression on tape 1 for the H-P computer. Type in the corrected peak heights of the blank and standards as x, the concentration for y. Obtain the alpha tocopherol values of the samples from the standard curve by using the corrected peak heights.

3. Determination of Antibodies

a. The passive hemagglutination titer of serum against a purified lipopoly-saccharide antigen of E.coli strain was determined by the micro titer method described by Herbert (1967) and Neter et al. (1956).

(1) Media: BBL trypticase soya agar and broth were prepared as previously mentioned.

(2) Antigen:

(a) E.coli strain was grown on broth for 18-20 hours.

(b) Transferred to plates and incubated for another 18-20 hours.

(c) The resulting growth was harvested in 25 ml PBS.

(d) Suspension heated in boiling H_2O for 1 hour.

(e) Supernate was obtained by centrifugation at 1500 rpm (HN-S Centrifuge, International Equipment Co Needham

Heights Mass USA.)

(f) Supernate is ready to use in HA test.

b. Red Blood Cells:

(1) Sheep RBCs taken from the jugular vein were collected in equal volume of Alsevier's solution.

(2) RBCs washed 3 times in PBS.

(3) To packed blood cells, add antigen so that RBCs concentration is 2.5%.

(4) Mix thoroughly by shaking.

(5) Incubate in water bath (HAAKE D, Lab. Line Magnestir Labline Instruments, Melrose Park ILL. USA) at 37°C for 30 minutes.

(6) Wash 3 times in PBS to remove excess antigen.

(7) Make to 1% suspension.

c. Test:

(1) Add 50 ul PBS in all wells of round bottom well microtiter plates.

(2) Add 50 ul of serum sample to well number 1.

(3) Use microdiluter to dilute sample (2 fold dilution)

(4) Add 50 ul of a 1% SRBC/antigen suspension.

(5) Red blood cell negative control (add 50 ul PBS and 50 ul RBC/antigen suspension).

(6) Leave undisturbed for 2 hours and read.

b. Hemmagglutination-inhibition (Beard and Wilkes, 1973)

(1) Reagents:

(a) phosphate buffered saline

(b) Alsevier's solution

(c) Red blood cells (RBC)

(i) Blood was obtained by heart puncture from White Leghorn chicks known to be free of antibodies against ND.

(ii) The blood was drawn into a syringe that contained Alsevier's solution in a volume of at least 1:1.

(iii) The RBC's were sedimented by centrifugation at 1200 rpm for 5 minutes.

(iv) The supernate was poured off and the cells washed 3 times in PBS.

(v) After the last centrifugation the supernate was aspired and a 1% suspension RBCs in PBS was prepared.

(d) Hemagglutinating (HA) antigen:

The proper dilution of the antigen to be used in preparing the antigen-saline mixture was determined as follows:

(i) Prepare 1:10 and 1:15 dilutions of

antigen in PBS.

(ii) Fill a microtest plate (clear, round bottom) with 50 ul of PBS in each well.

(iii) Add 50 ul of the 1:10 dilution to the first well in a row with a 50 ul microdiluter.

(iv) Add 50 ul of the 1:15 dilution to the 1st well in the 2nd row.

(v) Pass 50 ul quantities to achieve 2-fold dilutions.

(vi) Add 50 ul of 1% RBC suspension to each well.

(vii) Observe the plate after a control well that contains only 50 ul of PBS and 50 ul of RBC suspension exhibits a distinct "button" of RBCs in the bottom.

(viii) Record the last dilution in each row where there is complete hemagglutination and no button formation.

(ix) Obtain an average of the reciprocals of the highest agglutinated dilution in the row that began with 1:10 dilution with

the reciprocal of the next highest dilution that did not agglutinate in the row with the 1:15 dilution. This average is used to prepare an antigen-saline mixture that contains 10 HA units.

(2) HI procedure:

(a) In 1st top row of wells dispense 100 ul of antigen-saline mixture.

(b) In all other wells put 50 ul of antigen-saline mixture.

(c) Remove 10 ul from each top well with a 10 ul microdiluter, leaving 90 ul in the top wells.

(d) Add 10 ul of sera to be tested to each top well thereby making a 1:10 dilution of serum.

(e) Dilute the serum using 50 ul microdiluter to make 2-fold dilution.

(f) Incubate serum antigen mixture at room temperature for 20 minutes.

(g) Add 50 ul of RBC suspension to each well.

(h) Read results in 30-40 minutes.

APPENDIX "C"

Histopathological Technique

Histopathological Techniques (Luna, 1968)

A. Fixation

10% neutral buffered formalin was used.

B. Dehydrating, clearing, embedding, automatic processes:

1. Dehydrating

Remove all water from tissue. Series of changes through alcohols.

2. Clearing:

Xylene is used since it is mixable with alcohol and paraffin.

3. Impregnation:

Removal of clearing agents by infilteration with melted paraffin. 2 paraffin baths are used with a temperature of 56-58°C - melting point.

4. Embedding:

Tissue is embedded in melted paraffin with surface to be cut down.

5. Section are cut at 6u thickness with microtome and picked up on clean glass slide, drained and dried in 37°C oven over night.

6. Staining:

Hematoxylin and eosin were used. The progression of paraffin sections from de-

waxing xylene to staining are as follows.

a.	xylene	5 minutes
b.	xylene	5 minutes
c.	100% alcohol	5 minutes
d.	95% alcohol	5 minutes
e.	80% alcohol	3 minutes
f.	tap water wash	5 minutes
g.	Harris hematoxylin	5 minutes
h.	Distilled water rinse	5 minutes
i.	0.25% HCl	1-3 dips
j.	Tap water wash	5 minutes
k.	Eosin	3.5 minutes
l.	95% alcohol	rinse
m.	100% alcohol	5 minutes
n.	100% alcohol	5 minutes
o.	xylene	5 minutes
p.	xylene - until mounted with per-mount	

Harris hematoxylin

hematoxylin	1g
absolute alcohol	10ml
ammonium allum	20g
distilled H_2O	200ml
mercuric oxide	0.5g
acetic acid	5ml/100 ml stain

Eosin:

Stock eosin 1% in distilled H_2O

Stock phloxine 1% in distilled H_2O

Working solution:

stock eosin	25ml
stock phloxine	2.5ml
95% alcohol	195ml
glacial acetic acid	1ml

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Benso

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Bour

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Bro

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Ca

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