RED CELL FRAGILITY IN CHICKS
SUPPLEMENTED WITH VITAMIN E
AND SELENIUM

BY
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RED CELL FRAGILITY IN CHICKS SUPPLEMENTED
WITH VITAMIN E AND SELENIUM

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VITAMIN E AND SELENIUM

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AN ABSTRACT OF THE THESIS OF

Levon Karageuzian for Master of Science

Major: Animal Pathology

Title: Red cell fragility in chicks supplemented with vitamin E and selenium.

Diets containing low levels of vitamin E, selenium or both have been shown to produce physiological and nutritional disorders in several species. Among these manifestations increased erythrocyte fragility has been recognized.

In the present study straight run six day old chicks were fed a basal diet deficient in both vitamin E and selenium for nine days. Then they were subdivided into four randomized groups which continued to receive the same basal diet respectively: Unsupplemented, with vitamin E, with selenium or with both nutrients added at the recommended levels. All birds were individually tested at weekly intervals for: body weight, erythrocyte osmotic fragility, hematocrit and hemoglobin values. There was a highly significant increase in body weight gain per day for birds receiving vitamin E, selenium or both. No difference between deficient and supplemented birds was detected in relation to the other parameters.

This experiment was terminated when the chicks reached 23 days of age.
# TABLE OF CONTENTS

| LIST OF TABLES | vii |
| LIST OF FIGURES | viii |

<table>
<thead>
<tr>
<th>CHAPTER</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>II. REVIEW OF LITERATURE</td>
<td>3</td>
</tr>
</tbody>
</table>

- Vitamin E in relation to chicken nutrition and health | 3 |
- The antioxidant role of vitamin E in relation to cellular membranes | 5 |
- Relation of vitamin E to erythrocyte membrane fragility | 8 |
- Effect of vitamin E on growth | 12 |
- Effect of vitamin E on hemoglobin and hematocrit | 13 |
- Relation of selenium to chick nutrition and health | 14 |
- The antioxidant role of selenium in relation to cellular membranes | 16 |
- Function of selenium in relation to the erythrocyte membrane | 17 |
- Effect of selenium on growth, hematocrit and hemoglobin in chickens | 20 |

| III. MATERIALS AND METHODS | 22 |

- Experimental animal and design | 22 |
- Experimental diet | 22 |
- Blood sample collection | 23 |
- Erythrocyte fragility test | 24 |
- Statistical treatment of data | 24 |

| IV. RESULTS AND DISCUSSION | 26 |

| V. SUMMARY, CONCLUSION AND RECOMMENDATION | 32 |

| LITERATURE CITED | 33 |
LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Effect of vitamin E and selenium on chick growth</td>
<td>27</td>
</tr>
<tr>
<td>2. Effect of vitamin E and selenium on hematocrit values</td>
<td>30</td>
</tr>
<tr>
<td>3. Effect of vitamin E and selenium on hemoglobin values</td>
<td>31</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Effect of vitamin E and selenium on chick growth</td>
<td>28</td>
</tr>
</tbody>
</table>
I. INTRODUCTION

Vitamin E and selenium are trace nutrients that have been found essential in all domestic species examined. Both function as biological antioxidants in charge of countering the damaging affect of metabolic end products: The peroxide radicals. This they do through apparently different mechanisms whose end results are synergistically beneficial.

The antioxidant role of selenium is better understood in relation to hemoglobin within the erythrocyte. There the selenium dependent metallo enzyme glutathione peroxidase operates as a peroxide scavenger within the aqueous environment of the cytosol using glutathione as an H+ donor.

Although its mechanism of operation is less well understood. The antioxidant role of vitamin E is no less certain. It seems to function primarily; if not exclusively, within the lipoprotein environment of cellular membranes. There, by a process yet to be determined, it prevents peroxides from attacking the polyunsaturated fatty acids whose presence is required for the structural integrity and normal activity of these membranes.

No wonder, in view of the involvement of vitamin E and selenium in such fundamental functions, that their deficiency would lead to the complex clinical syndromes conventionally described in the literature: encephalomalacia, nutritional muscular dystrophy and exudative diathesis.
Less clearly established and still a matter of controversy is their relation, singly or combined, to growth and the hematologic status of deficient animals. This is particularly true for the chicken about which there is scanty information.

In the present study the objectives were as follows:

1. To determine the effect on young chick erythrocyte susceptibility to hypotonic NaCl solutions of the single deficiencies and their combination.

2. To examine whether these deficiencies lead to detectable changes in the hematocrit and hemoglobin values of these chicks.

3. Finally to record body weight gains and compare how growth rate is affected by the availability by one or the other of the two nutrients or their combination.
II. REVIEW OF LITERATURE

**Vitamin E in Relation to Chick Nutrition and Health**

Vitamin E was discovered in 1922 by Evans and Bishop and was isolated as \( \alpha \)-tocopherol by Evans and Emerson in 1936. The prefix \( \alpha, \beta, \gamma \) were used to indicate the various forms of tocopherols. D-\( \alpha \) tocopherol was biologically the most active.

Studies made in experimental animals of various species in time led to the recognition of vitamin E deficiency as the cause of several pathological conditions.

The deficiency signs occur most readily in growing chicks, rather than in mature chickens. In general vitamin E is required for fertility and for normal reproduction performance in the hen; however, few studies have been made on its role in the reproductive process in poultry. Testicular degeneration and reduction in fertilizing capacity was observed. Restoration of fertility with vitamin E was observed by Arscott (1965). Similar results were obtained by Machlin (1962).

In young chicks vitamin E deficiency may result in body lipid oxidation and erythrocyte hemolysis. In the case of acute deficiency the birds show one or more of the following manifestations: encephalomalacia, exudative diathesis or muscular dystrophy. An adequate supplementation with vitamin E will prevent all above clinical forms.

Several different metabolic functions have been attributed to vitamin E in view of the diversity of tissues affected in vitamin E
deficiency. Scott (1969) and Morton (1970) have summarized these functions, some of which are (1) as a biological antioxidant (2) a tissue respiration agent (3) the synthesis of ascorbic acid (4) the synthesis of ubiquinone (5) protein and sulfur amino acid metabolism. The way in which vitamin E is involved in these functions remains to be determined.

Much of the interest has concentrated on its role as antioxidant in relation to cellular membranes or tissues, and its protective or nutritive effect has been explained through this role. Bell and Freeman (1972), stated that there is a controversy whether or not its antioxidant properties can entirely account for its biological activity.

Reports in this respect are conflicting. Scott and Stones (1961), thought that the protective effect of the antioxidant is exerted directly in the tissues, and is not the indirect result of preventing the formation of toxic oxidation products in the food. The same idea was proposed by Tappel (1965). Some of the experimental findings, however, do not fit this hypothesis. For instance there was no evidence for increased peroxidation in chicks deprived of vitamin E. Diplock et al. (1967), and Bunyan et al. (1967), were unable to detect an increased rate of destruction of tocopherol in either cerebellum or brain of chicks receiving enough poly unsaturated fatty acids to produce encephalomalacia, although a single dose of 1 mg of D-α tocopherol delayed the onset of encephalomalacia. In muscular dystrophy it was found that the dystrophic muscles contained vitamin E twice as high as in muscles from control birds. However
both groups of writers concluded that although there is close relation
between vitamin E and the metabolism of unsaturated fats it cannot be
interpreted solely in terms of the antioxidant function of vitamin E.

The amount of vitamin E needed by the chicken varies because
of the marked effect of other nutrients on the need for tocopherol.
Thus no general estimate of the vitamin E requirement can be formulated
which is quantitatively meaningful. Scott (1969), after reviewing all
reports concluded that the amount of vitamin E is variable with the
type of food given namely with the kind and amount of fatty acids in
incorporated in the food, with the growth stage of the chicken, and with
the amount of selenium found in the feed. However, 10 IU/kg of diet is
recommended for growing chickens (Nutritional Requirements of Poultry,
1971).

The Anti-oxidant Role of Vitamin E in Relation to Cellular Membranes

The biological function of vitamin as a lipid antioxidant
has been a topic of research for over twenty years, specially oxidations
that result in cellular damage such as the hemolysis of erythrocytes.
Morton (1970), considers this phenomenon as the earliest sign of cellular
damage in \( \alpha \)-tocopherol deficient animals. The first anti-oxygenic
activity was demonstrated by Moore (1940), and Dam (1957), who proved
that synthetic antioxidants are capable of substituting for dietary
tocopherol in preventing vitamin E deficiency manifestations. Interest
in vitamin E as antioxidant started after these experiments. Molenar
et al. (1972) after reviewing the litterature about the antioxidant
activities of \( \alpha \)-tocopherol concluded that four hypotheses on the functions
of vitamin E as antioxidant in relation to cellular membranes have been
suggested.

a. Vitamin E as the non-enzymatic biological antioxidant.
b. Vitamin E as a factor in enzyme dependant lipid peroxidation.
c. Vitamin E as a factor in biological oxidation or oxidative phosphorylation.
d. Vitamin E as a membrane stabilizer with redox capacities.

The first theory has found strong advocates in Tappel (1962), and Howitt (1965).

Tappel (1962), after reviewing several references stated the following:

"... Free radical chain reactions are the basic mechanism in many deteriorative reactions... this chain reaction can be effectively inhibited by antioxidants... animals that contain the very oxygen-labile polyunsaturated fatty acids (P.U.F.A.) require lipid antioxidants, and this biological essential of lipid antioxidant is filled by vitamin E... the chemical basis of vitamin E function is its reaction with free radical intermediates of lipid peroxidation and with peroxides."

According to Tappel there are two routes of direct experimental evidence for the role of vitamin E as a biological lipid antioxidant.

1) Replacement of vitamin E with structurally dissimilar lipid antioxidant.

2) Direct measurement of lipid peroxidation in vitamin E deficient animals.

For the first part both Bieri et al. (1960), and Machlin (1959),
provided evidence where some antioxidants could replace vitamin E to a certain extent but the replacement could not prevent totally the occurrence of the deficiency signs. For the second, increased peroxidation of lipid was measured in vitamin E deficient animals, the tissues involved being liver, heart and lumbar fat (Tappel, 1962). However, the second theory does not apply to the tissue obtained from brain. Diplock et al. (1967), and Bunyan et al. (1967), could not measure increased peroxidation in the brain of chickens suffering from encephalomalacia. At this stage we might assume that the antioxidant function of vitamin E is confined to certain tissues.

Theories under B and C are applicable to most cellular membranes except that of erythrocytes. Bleri and Pouka (1970), and Molenar et al. (1972) in their reviews suggested that vitamin E might intervene in electron transport systems. Peroxidation could damage the membrane bound enzyme system which is involved in electron transport as is implied in B. With regard to oxidative phosphorylation the role of vitamin E would be confined to mitochondria and endoplasmic reticulum. This vitamin is predominantly localized in the inner mitochondrial membrane where the other members of the respiratory chain are also located. As to the stabilizer function of vitamin E the best explanation comes from Lucy (1972). She suggested that vitamin E stabilized membranes by virtue of specific physicochemical interaction between its chromanol ring and the double bonds of P.U.F.A. Molenar et al. (1972), in their review consider two more observations about vitamin E in relation to cellular membranes. First, the positive effect of vitamin E on the ultra structure of membranes,
second, the increased permeability of vitamin E deficient cells, specially to $K^+$ when the permeability becomes marked lysosomal membranes are affected and hydrolytic enzymes are released which results in cellular damage.

Relation of Vitamin E to Erythrocyte Membrane Fragility.

The main interest in vitamin E as relating to erythrocyte membrane fragility started with Rose and Gyorgy (1950). When alloxan was injected to animals with vitamin E deficiency, hemoglobinuria was noticed and hemolysis occurred when erythrocytes were tested with dialuric acid. Feeding the animals with vitamin E for 3 days was enough to give complete resistance to the cells. The conclusion they had was that vitamin E reacted with some intermediate of the system, a free radical or peroxide which is actually the hemolyzing agent.

Rose and Gyorgy (1952), further investigated the role of alloxan and stated that alloxan is active in vivo because the reducing system of the body converts it in part to dialuric acid. They later on used hydrogen peroxide to determine the potency of different kinds of tocopherols and thus confirmed that $\alpha$-tocopherol is the most active form. Rose makes the following statement about hydrogen peroxide hemolysis.

"In our test with hydrogen peroxide we found as previous workers had indicated that deficiency of vitamin E was not essential for hemolysis, cells obtained from vitamin E treated rats could be almost completely hemolyzed. However, vitamin E deficient cells are much more readily hemolyzed."
Rose assumed hydrogen peroxide was the intermediate for hemolysis and not the sole hemolyzing agent. Experiments continued on the same trend, modifications were introduced, but the relationship of vitamin E and other antioxidants in preventing hemolysis. Both N-N-diphenyl-P-phenylenediamine (D.P.P.D.) and butylated hydroxy toluene (B.H.T.) could not replace vitamin E. Thus they suggested that the role of vitamin E in preventing hemolysis may be related to enzymic or other type of catalytic process and not a simple combination with peroxide. Tappel (1962), after reviewing forty articles came to conclusion that the chemical basis of vitamin E function is its reaction with free radical intermediates of lipid peroxidation and peroxide.

Jacob and Lux (1968) were pioneers in interpreting the relationship of vitamin E and erythrocyte membrane fragility. They describe lipid peroxidation in erythrocyte membrane as an alteration in the lipid layer due to cleavage of unsaturated fatty acids at their double bonds. They emphasized that intracellular metabolism was not affected in deficient red cells. In a further attempt they analyzed the specific phospholipid which was oxidized. They reported that a striking decrease in the phosphatidyl ethanolamine was observed in E deficient cells but not in E supplemented ones while both sphingomyelin and cholesterol remained unchanged. They presumed that the cleavage of phosphatidyl ethanolamine results from destruction of its fatty acid.

The explained the role of \( \alpha \)-tocopherol as follows:

"... Examination of the molecular structure of vitamin E suggests mechanisms by which this substances might inhibit red cell
membrane damage. Alpha-tocopherol contains a long chained fatty acid which imparts solubility to the vitamin in the lipid milieu of membranes, the remainder of the molecule is an aromatic substituted phenol. The latter by virtue of its unsaturated resonating structure can trap free radicals that exist in the presence of H$_2$O$_2$ or its breakdown products while itself it is stabilized by resonance...

In the absence of vitamin E H$_2$O$_2$ is free to react with other double bonds in the cell membrane ... Because phosphatidylethanol amine contains the greatest proportion of unsaturated fatty acid ... it is vulnerable to destruction by H$_2$O$_2$ degradation of it is followed by membrane disorganization forming holes large enough to cause leakage." This was the first attempt of its kind since 1950 in explaining the mechanism of vitamin E action. Previous experiments were only concerned in stabilizing the method and giving short range explanation in terms of lipid peroxidation.

Hayes and Nielson (1969) proposed that vitamin E might accelerate the removal of abnormal lipids such as polymerized P.U.F.A. of erythrocytes rather than being involved only in antioxidant process. O'Brien and Little (1969) suggested that the role of vitamin E might be to decrease the amount of hydroperoxide formation. The same idea is proposed by Bobath (1969), who after reviewing several articles concluded that prevention of hydroperoxide formation might be its main function. Through the 1960's experiments were carried in order to specify the specific fatty acid affected by the deficiency. Animals were fed with different fatty acids and $\alpha$-tocopherol intake was controlled. The amount of vitamin E needed to give maximum resistance
to the cells depended upon the type of fatty acid incorporated in the diet. Bieri and Pouka (1970) established that the more linoleic acid intake the more vitamin E needed. At this stage the postulate of Jacob and Lux (1968) that phosphatidyl ethanolamine (P.E.) is the primary fatty acid which is oxidized was seriously challenged by Batt and Winterbourn (1970) who studied the incorporation of plasma fatty acid into human erythrocytes. They found that younger R.B.C., incorporated plasma fatty acids into P.E. much less efficiently than did older R.B.C. to them, the considerable amount of reticulocytosis exhibited by E deficient rabbits suggests that most of the fatty acids incorporated from plasma into membrane phospholipids would be found in the phosphatidyl choline fraction. They considered this effect was enough to outweigh the phenomenon described by Jacob and Lux of greater incorporation of fatty acid into P.E. Further investigations along this line led to contradictions. Heikila et al. (1971) analyzed the R.B.C. phospholipids obtained from vitamin E deficient rats, and after oxidation, losses were found in the ethanolamine, choline and serine phosphoglyceride fractions. So far we see that up to 1970's the role of vitamin E as a nonenzymatic biological antioxidant was favored.

Lucy (1972) suggested a structural role for vitamin E in the control of membrane stability and permeability.

"... Vitamin E stabilizes membranes by virtue of specific physiochemical interactions between its phytyl side chain and the fatty acid chains of polyunsaturated phospholipid particularly those derived from arachidonic acid. Studies with molecular models showed that, the methyl groups at C-4 and C-8 of \( \alpha \)-tocopherol can fit into
pockets provided by the 1st and 3rd is double bonds from the arachidonyl carboxyl group... In the complex thus formed the Ho group of the chromanol ring of α-tocopherol and the polar groups of the phospholipid all lie at the same end of the complex, where they can participate in hydrophilic interactions at the surface of membrane having a lipid bilayer, thus by this method vitamin E can stabilize membranes."

Lynda et al. (1974), explained the role of vitamin E in a different fashion. In their investigation they observed that fat from deficient rabbits contains significantly less arachidonic acid than does that from control animals. They suggested that perhaps vitamin E is necessary for the biosynthesis of arachidonic acid from linoleic acid, via eicosadienoic acid path. In all groups values for eicosadienoic acid content were higher in R.B.C. of control rabbits than R.B.C. of corresponding deficient ones. They suggested that this effect might be due to an interruption of the conversion of linoleate to eicosadienate in the absence of vitamin E. Perhaps vitamin E does not itself participate in the reaction but some intermediate product whose absence might interfere with the biosynthesis of eicosadienoic acid.

At this stage we may conclude that no clear understanding of the biological function of vitamin E has been obtained as relating to cellular membranes stability.

Effect of Vitamin E on Growth

There has been a general consensus, that vitamin E has a deep effect on growth of animals although the extent of this effect still
is in question. Bieri et al. (1960) found no difference between animals supplemented with vitamin E and the deficient ones. In a review about the effect of selenium and vitamin E on growth Scott (1969), concluded that chicks receiving high amount of D-α-tocopherol acetate, 1000 IU/kg of diet, without selenium grew at much slower rate than those receiving selenium alone. Thomson and Scott (1969), and Cantor et al. (1975) reached to the same conclusion. Nevertheless, all the above writers recognized that vitamin E is essential for normal growth, and supplementation with antioxidants and selenium in order to replace vitamin E totally was unsuccessful. Cheeks and Shull (1972), reported that rats depleted from vitamin E and selenium were able to restore their weight when supplemented with vitamin E.

It is clear that a deficiency in vitamin E will affect the normal metabolism of the body thus affecting the normal growth. However the extent of this effect is still in question.

Effect of Vitamin E on Hemoglobin and Hematocrit

Reports on the effect of vitamin E on chick hematocrit and hemoglobin are unavailable while reports on other animals in this respect are scarce because anemia is not a regular feature of tocopherol deficiency. One report by Bieri et al. (1960), mentions that there is no difference in hemoglobin and hematocrit values for vitamin E supplemented and deficient chicks at 19 weeks of age. Dinning and Day (1957), observed a distinct depression of hemoglobin levels in deficient monkeys. A similar observation was made by Porter et al. (1962) who reported low hemoglobin levels, low erythrocyte counts and corresponding reduced packed cell volumes with
depressed porphyrin and heme synthesis in vitamin E deficient monkeys.
Darby (1968), in a review about the effect of tocopherol on anemia reported low hematocrit values in trout deficient in tocopherol and increased reticulocytosis in pigs. He concluded that although erythropoiesis is accelerated it is, however, ineffective. Fitch (1968) coming to the same conclusion as Dining and Day (1957) and Porter et al. (1962), stated that monkeys deficient in vitamin E showed progressive weight loss and anemia, decrease in hemoglobin concentration, and the red cell precursors were abnormal in morphology and function again deducing that anemia is due to ineffective erythropoiesis. The situation was not the same with Rotruck et al. (1972). In their work with rats, there was no difference in hematocrit due to dietary treatment of the animals, the treatments were with vitamin E and selenium.

In conclusion the hematologic effects of vitamin E are not well established and might be species dependent.

Relation of Selenium to Chick Nutrition and Health

Selenium was discovered by Berzelius about 150 years ago. It is only its toxicity that was known at first as the cause of alkali disease in cattle, and other livestock malformations in chickens, Scott (1969). In 1957 it was discovered that selenium at 0.05 to 0.2 p.p.m. is an essential nutritional element, which renewed interest in it (Scott, 1969). In 1955 Scott and associates reported that brewer's yeast contains a factor which replaced vitamin E in the prevention of exudative diathesis. Further experiments were conducted and the role of selenium is preventing vitamin E
deficiency manifestations, such as muscular dystrophy and exudative diathesis, were investigated (Scott, 1969). NescheIN and Scott (1958) concluded that selenium is mainly involved in prevention of exudative diathesis and is effective at very low levels of supplementation of the order 0.1 p.p.m. According to the same authors the role of selenium in relation to muscular dystrophy was limited to the process of vitamin E absorption and its utilization. Thomson and Scott (1969) proved that vitamin E or selenium could not replace each other totally, although they have a sparing effect on each other. He reported that selenium deficiency does produce a specific deficiency syndrome even in the presence of high dietary levels of vitamin E. This syndrome is characterized by retarded growth, poor feather development and fibrotic degeneration of the pancreas. Tappel (1965) reported that both vitamin E and selenium reduced the peroxidazability of cellular lipids. The notion was that selenium is incorporated into animal proteins to form selenoproteins which function as antioxidant. Hamilton and Tappel (1963) concluded that selenium is specifically incorporated into the proteins of liver. On the other hand Scott (1969) reported that selenium is firmly bound to amino acids and strongly associated with cystine and methionine, which led to speculations that it might replace sulfur in cystine and methionine.

Recently the relationship of selenium and glutathione peroxidase has attracted much attention. Rotruck and Pope (1973) found that selenium was an integral part of glutathione peroxidase without which glutathione fails to function as a peroxide receptor. Scott (1969), stated that selenium has an effect upon the absorption and retention
of D-α-tocopherol. Chicks receiving a basal diet low in selenium had one percent less plasma vitamin E than chicks receiving the same diet with adequate selenium content. Although the synergistic effect of vitamin E and selenium is clear the mechanisms of their interaction remain to be determined.

The Anti-oxidant Role of Selenium in Relation to Cellular Membranes

The fact that vitamin E and selenium work synergistically as biological antioxidant, their inter-relationship in this respect has become the object of investigation only over the past ten years. Zaklin et al. (1962) reported extensive evidence regarding the in vitro prevention of lipid peroxidation in liver, kidney and heart tissue from selenium fed chickens. The inclination at that time was to think that antioxidants formed in the chicken may be selenoamino acids or selenoproteins. Tappel (1962), reviewing the existing literature about selenium as an antioxidant concluded the following:

"... The selenium antioxidants have not been identified but it is probably that many selenium compounds are involved in the antioxidant reaction, considering the chemical similarities of selenium and sulfur... probably antioxidation reaction would be similar to those discussed for the sulfur amino acids, namely:

1. Reaction with free radical intermediates of lipid peroxidation to break chain reaction.

2. Decomposition of lipid peroxides.

3. Reduction of other primary antioxidants like tocopherol.

Evidence from the study of selenium antioxidant favors reactions of the first type."
Tappel (1965), reported that vitamin E and selenium reduced the peroxidazability of cellular lipids. Rotruck et al. (1972) investigated the relation of selenium to glutathione peroxidase, and concluded that the enzyme eliminates hydrogen peroxide in erythrocytes and preserves the cell from oxidative damage. This relationship will be discussed later.

In general, a deficiency in both vitamin E and selenium should enhance lipid peroxidation and favor damage to membranes. This may result in cell lysis and disruption of subcellular structures and lysosomes. Such disorganization of membrane structures would account for the tissue changes specially when one considers all hydrolytic enzymes set free in the tissues.

**Function of Selenium in Relation to the Erythrocyte Membrane**

The relation of selenium to erythrocytes was analysed through experiments involving glutathione (G.S.H.) peroxidase which catalyzes the breakdown of hydrogen peroxide.

G.S.H. peroxidase was first detected by Mills and Randal (1958) in erythrocytes lysate which can protect hemoglobin from oxidative breakdown. He was first to account for the in vitro protective role of glucose which maintains glutathione in the reduced stage. G.S.H. peroxidase catalizes the breakdown of \( \text{H}_2\text{O}_2 \) to \( \text{H}_2\text{O} \) a reaction in which G.S.H. acts as a hydrogen donor. Cohan and Hochstein (1961) have shown that cells unable to generate an adequate supply of reduced glutathione undergo hemolysis. On the other hand impaired generation of NADPH leads to a depression in the production of reduced glutathione.

In an attempt to evaluate the primary scavenger of intracellular \( \text{H}_2\text{O}_2 \)
Cohan and Hochstein (1963), established that glutathione peroxidase was the enzyme involved and not catalase. They also indicated that progressive oxidation of hemoglobin due to $H_2O_2$ was accompanied by increasing osmotic fragility eventually leading to lysis even in isotonic medium. The erythrocytes used here were human cells. Moreover, they concluded that glucose protects red blood cell from hemolysis by its ability to provide a source for NADPH, through glucose six-phosphate dehydrogenase, which subsequently leads to the formation of G.S.H. In 1969 O'Brien in his review concluded that the G.S.H. peroxidase plays an important role in protecting membrane lipids from oxidative disintegration. Up to that date there was no indication about role played by selenium. Investigators agreed that vitamin E and selenium have synergistic effects with respect to erythrocyte membrane. The role of vitamin E was partially clarified but that of selenium remained uncertain. Rotruck et al. (1971) studying autohemolysis in vitro found that the protective effects of dietary selenium were dependant on the addition of glucose to the medium containing the erythrocytes where as vitamin E protects the cells without need for glucose. They concluded that the mechanism whereby selenium stabilized R.B.C. was specific and distinct from that of vitamin E. In a further investigation Rotruck et al. (1971) proved that G.S.H. concentration was higher in the erythrocytes of selenium deficient animals, than those of supplemented ones. They deduced that selenium was not necessary for the generation of G.S.H. but rather for its utilization. Later on Rotruck et al. 1972 using rat erythrocytes proved that selenium like vitamin E could protect the
R.B.C. membrane but selenium unlike vitamin E could also prevent the oxidation of hemoglobin. These authors make the following comments:

"... The specific and primary role of selenium and its relation to glutathione in protecting against oxidative damage of erythrocytes remains subject to speculations, specific enzyme system which utilizes glutathione catalyze the destruction of hydrogen peroxide or fatty acid hydroperoxide... A catalytic role of selenoamino acids or other low molecular weight forms of selenium in destroying peroxides, or in repairing or preventing oxidative damage to sulphydryl containing enzymes could explain the effect of selenium in preventing oxidative damage to erythrocytes, alternatively one or more of the enzymes involved in destroying peroxides or maintaining sulphydryl groups may contain selenium as an integral and necessary part of the enzyme, our preliminary evidence suggest that the second alternative is more likely to occur."

Noguchi et al. (1973) In their study about vitamin E and selenium in relation to lipids stated that glutathione peroxidase is associated largely with the aqueous phase of the cell or cytosol, accordingly and peroxides that appear inside the cells are immediately destroyed by G.S.H. peroxidase within the cytosol. If the diet is low in selenium, vitamin E within the membrane takes over. This was the first attempt to explain the distinct roles of vitamin E and selenium. Rotruck and Pope (1973) further explored the association between selenium and G.S.H. and came to the conclusion that selenium is an integral part of G.S.H. peroxidase. Hafeman et al. (1974)
suggested that selenium is incorporated into erythrocytes G.S.H. peroxidase only during erythropoiesis which explains the failure of selenium in preventing hemolysis when added in vitro. Cheek and Shull (1972) and Hafeman et al. (1974) demonstrated that peroxidase activity is directly proportional to selenium level in plasma.

Selenium then being an integral part of G.S.H. peroxidase functions as an antioxidant in the form of selenoprotein or a selenium dependent enzyme which is the actual H₂O₂ scavenger protecting hemoglobin from oxidation. Although the mode of action of vitamin E as an antioxidant is still unclear the evidence is that vitamin E operates within the lipoprotein environment of cellular membranes which it protects from the damaging effects of peroxides thus complementing the role of selenium.

Effect of Selenium on Growth Hematocrit and Hemoglobin Chickens

In a review about the effect of selenium on growth, Scott (1969) concluded that dietary selenium alone is superior to vitamin E alone in promoting growth in chicken and nearly was as good as vitamin E and selenium combined. Cantor et al. (1975), drew the same conclusion when they found that chicks supplemented with 0.1 p.p.m. of selenium/kg diet without vitamin E grew better than a similar group of birds receiving as much vitamin E as 1000 IU/kg of diet without selenium.

The observation that vitamin E and selenium together resulted in the same growth as selenium alone was made both by Scott (1969) and Rotruck et al. (1972).

It is clear that both vitamin E and selenium improve growth. However, the above experiments suggest that their mode of action is
distinct, although no explanation of this difference is yet available.

No reports are available on selenium regarding its effect on hematocrit and hemoglobin values in chickens Rotruck et al. (1972) found no difference in hematocrit value between rats receiving vitamin E and selenium as opposed to deficient controls.
Experimental Animal and Design

Day old chicks directly imported from a commercial hatchery were kept in electrically heated brooders at a temperature of 38°C and were fed a normal diet for six days. On the 7th day they were shifted to a basal diet deficient in vitamin E and selenium for a period of nine days. Then they were divided into four groups according to weights, the range of the first group was 50-60 g, of the second 60-70 g, of the third 70-80 g, and of the fourth 80-90 g. From each group 3 chicks were chosen randomly and incorporated into one composite group, such that the newly formed four groups contains each 12 chicks chosen randomly from the above four groups. This method was followed in order to randomise weights among the four experimental groups. The latter comprised a control group which continued to be fed the deficient basal diet (d.b.d.) described later on, a selenium supplemented group receiving d.b.d. fortified with sodium selenite, a vitamin E supplemented group and a group receiving both selenium and vitamin E.

Experimental Diet

A purified diet formulated by Scott (1969), with small modifications was used as a basal diet. Its composition was as follows:

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<thead>
<tr>
<th>Ingredient</th>
<th>Percentage</th>
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<tbody>
<tr>
<td>Isolated soybean protein</td>
<td>25%</td>
</tr>
<tr>
<td>Corn oil</td>
<td>4%</td>
</tr>
<tr>
<td>Cellulose</td>
<td>3.32%</td>
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Corn starch 50%
D-L-Methionine 0.6%
Choline chloride 0.25%
Vitamin premix (without vitamin E) 1.20%
Mineral premix (without selenium) 5.63%
Antioxidant (B.H.T.) 10%

B.H.T. was chosen for antioxidant in vitro because it does not substitute for vitamin E in vivo (Bunyan et al. 1960). After dividing the basal diet into four equal amounts. The first part was kept as such without vitamin E and selenium, to the second vitamin E was added at 20 mg/kg diet, that is twice the recommended dietary amount (Nutrient Requirements of Poultry, 1971). To the third portion selenium was added at 0.5 mg of Se/kg diet in the form of sodium selenite, the fourth aliquot contained vitamin E and selenium at the above mentioned levels.

**Blood Sample Collection**

For the first two weeks blood was collected from the jugular vein using 22 gauge needles, from the third week on 21 gauge needles were used to collect blood from the wing vein. Heparin was added as anti-coagulant at 150-200 U.S.P. unit per 3 ml of blood.

Blood collection was done from eight animals per group, at weekly intervals, such that the first sampling for each group preceded the dietary changes as applicable. All groups were treated similarly. Directly after collection of blood hematocrit and hemoglobin tests were done. Then the blood samples were centrifuged at 2000 R.P.M. for 5 minutes, the plasma and theuffy coat were
removed by suction into pasteur pipets, the packed cells were washed with 4 ml of 0.9% NaCl solution and repacked at 2000 R.P.M. for 5 minutes (L.E.C. centrifuge, size 2, Model K). The supernatant was removed, the packed cells suspended at 1:20 ratio in 0.9% NaCl solution, and this suspension used for the fragility test procedure.

**Erythrocyte Fragility Test**

To test erythrocyte fragility 0.5 ml of 5% R.B.C. suspension were added to 7.0 ml of each of the following NaCl concentrations expressed in grams/100 ml: 0.9, 0.76, 0.68, 0.60, 0.52, 0.48, 0.44, 0.40, 0.36, 0.32, 0.28, 0.24, 0.20, and 0. The required amounts of NaCl solution and R.B.C. suspension were dispersed in clean pyrex serology tubes which were then gently shaken for mixing. The mixtures were held one hour at 37°C in an incubator then centrifuged at 200/R.P.M. for 3 minutes. The supernatant clear fluid samples were individually transferred to the appropriate cuvettes and their optical density read at 540 n.m. in a spectronic 20 instrument (Bauch and Lomb N 0904 Analytic System Division). A 0.9/100 ml saline solution served as blank whereas the last tube in the series presented above served as the 100% hemolysis control. The rate of hemolysis per tube was calculated as follows:

\[
\% \text{ Hemolysis} = \frac{O.D. \text{ Sample}}{O.D. \text{ of 100\% hemolysis}} \times 100
\]

**Statistical Treatment of Data**

The statistical analysis of the data was performed according to the methods described by Senedcor and Cochran (1971). Hematocrit and hemoglobin values were converted into angular values and their
analysis done by a two way classification method which takes into account both treatment and duration of treatment.

Mean weight gains/day were tested by the Duncan Multiple Range Method.
IV. RESULTS AND DISCUSSION

In Table I and Figure I are summarized the results which illustrate the effect on growth of vitamin E and selenium supplementation either singly or in combination. It is evident from the highly significant increased growth rate in the supplemented groups that the basal diet was deficient in both nutrients.

Although the difference in growth rate between the group receiving vitamin E alone and those receiving selenium was not significant, there is no doubt that the addition of selenium supported better growth. It is not clear whether the observed difference would have become significant had the experiment lasted a longer time.

Paradoxically and as may be seen from the curves in Figure I, selenium supplemented singly stimulated a faster and earlier growth response than the dual Se-vitamin E supplementation. Determinations of carcass composition might have been useful in the analysis of this phenomenon.

The same curves in Figure I show that toward the terminal stage of the experiment the group receiving both selenium and vitamin E tended toward a growth rate faster than that of the group supplemented with selenium only. It would be interesting to conduct a similar experiment for a longer duration and find whether the observed trend would confirm itself. Such an experiment would be all the more important that the available information in this respect is conflicting. (Scott, 1969; Thomson and Scott (1969); Cantor et al., 1975).
### Table 1: Effects of Vitamin E and Selenium on Chick Growth

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Basal Diet + Basal Diet + Selenium</th>
<th>Basal Diet + Basal Diet + Vitamin E and Selenium E</th>
<th>Basal Diet + Basal Diet + Vitamin E and Selenium E + Basal Diet</th>
<th>Gain/day (g)</th>
<th>Gain/day (g)</th>
<th>Gain/day (g)</th>
<th>Overall Gain (g)</th>
<th>% Gain</th>
<th>% Gain</th>
<th>% Gain</th>
</tr>
</thead>
<tbody>
<tr>
<td>18 + 0.3%</td>
<td>14.2 ± 0.4</td>
<td>11.7 ± 0.5</td>
<td>9.0 ± 0.1</td>
<td>1.85 ± 0.1</td>
<td>(a)</td>
<td>3.7%</td>
<td>14</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>21 ± 0.2%</td>
<td>18.8%</td>
<td>16.7%</td>
<td>16.1%</td>
<td>6.57 ± 0.9</td>
<td>14</td>
<td>3.5%</td>
<td>14</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15 ± 0.2%</td>
<td>6.4%</td>
<td>6.4%</td>
<td>6.4%</td>
<td>6.4%</td>
<td>14</td>
<td>3.5%</td>
<td>14</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4.8 ± 0.2%</td>
<td>3.7%</td>
<td>16.1%</td>
<td>16.1%</td>
<td>6.57 ± 0.9</td>
<td>14</td>
<td>3.5%</td>
<td>14</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean weight</td>
<td>351.2 ± 12</td>
<td>371.5</td>
<td>378.8</td>
<td>14</td>
<td>3.7%</td>
<td>14</td>
<td>14</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gain/day</td>
<td>22.3 ± 0.2</td>
<td>30.3 ± 0.1</td>
<td>21 ± 0.4</td>
<td>7.4 ± 0.2</td>
<td>(a)</td>
<td>6.4%</td>
<td>14</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% Gain</td>
<td>60.7%</td>
<td>94%</td>
<td>64.6%</td>
<td>64.6%</td>
<td>14</td>
<td>64.6%</td>
<td>14</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean weight</td>
<td>412 ± 11</td>
<td>435 ± 15</td>
<td>478 ± 11</td>
<td>344 ± 10.4</td>
<td>(a)</td>
<td>64.6%</td>
<td>14</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% Gain</td>
<td>296 ± 8</td>
<td>223 ± 9</td>
<td>230 ± 9</td>
<td>290 ± 15.4</td>
<td>(a)</td>
<td>64.6%</td>
<td>14</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Values in grams ± S.E.*
Figure 1. Effect of vitamin E and selenium on chick growth.
In Table 2 and 3 are summarized the hematologic values recorded in this experiment. Neither the dual deficiency nor the single ones had any detectable effect on hematocrit or erythrocyte fragility. The present findings are in general agreement with previous reports (Bieri et al., 1960, Rotruck, et al., 1972). Our erythrocytes were examined for stability in hypotonic NaCl solutions. There is no assurance that the two methods read the same parameter in erythrocyte membranes as affected by the nutritional deficiencies under examination. It would be desirable to repeat the same experiment applying the two tests for comparison.

A final observation may be made here regarding the selection a method to test a diet for its adequacy in vitamin E or selenium content. Although increased red cell fragility has been regarded as the most sensitive test procedure to detect the deficiency, the present results suggest that growth rate of very young chicken could be the choice biological test to be used because of its great sensitivity.
Table 2. Effect of vitamin E and selenium on hematocrit values (Mean ± S.E.).

<table>
<thead>
<tr>
<th>Duration of experiment (days)</th>
<th>Basal diet</th>
<th>Basal diet + vitamin E</th>
<th>Basal diet + selenium</th>
<th>Basal diet + vitamin E and selenium</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>25.4 ± 0.7</td>
<td>23.2 ± 0.6</td>
<td>27.2 ± 0.6</td>
<td>24.7 ± 0.8</td>
</tr>
<tr>
<td>7</td>
<td>26.9 ± 0.9</td>
<td>26.6 ± 0.9</td>
<td>23 ± 0.5</td>
<td>26.8 ± 0.8</td>
</tr>
<tr>
<td>14</td>
<td>25.3 ± 0.8</td>
<td>28.8 ± 0.3</td>
<td>25.4 ± 0.5</td>
<td>25.4 ± 0.7</td>
</tr>
</tbody>
</table>
Table 3. Effect of vitamin E and selenium on hemoglobin values (Mean ± S.E.).

<table>
<thead>
<tr>
<th>Duration of experiment (days)</th>
<th>Basal diet</th>
<th>Basal diet + vitamin E</th>
<th>Basal diet + selenium</th>
<th>Basal diet + vitamin E and selenium</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>9.5 ± 0.4</td>
<td>8.10 ± 0.4</td>
<td>9.5 ± 0.2</td>
<td>9.0 ± 0.2</td>
</tr>
<tr>
<td>7</td>
<td>10.0 ± 0.4</td>
<td>9.4 ± 0.5</td>
<td>8.0 ± 0.3</td>
<td>10.6 ± 0.5</td>
</tr>
<tr>
<td>14</td>
<td>9.3 ± 0.6</td>
<td>10.3 ± 0.4</td>
<td>8.3 ± 0.3</td>
<td>10.4 ± 0.5</td>
</tr>
</tbody>
</table>
V. SUMMARY, CONCLUSION AND RECOMMENDATION

Two week old birds in four experimental groups of eight were fed respectively a doubly deficient diet lacking selenium and vitamin E, with selenium or with both nutrients after a period of nine days on the doubly deficient semipurified diet. Body weights and blood samples were taken at weekly intervals through the fourth week of age. Osmotic erythrocyte fragility, hematocrit and hemoglobin values were determined. A highly significant growth rate difference between the supplemented groups and the unsupplemented one was recorded. No difference in red cell fragility, hemoglobin or hematocrit values was detected. It is clear from the present observations that both nutrients should be available in poultry diets for optimum performances.

It will be of interest that future work be directed at the following:

1. The experimentation period should be prolonged in order to confirm that a dual supplementation is better than selenium alone.

2. The conventional $H_2O_2$ test should be compared with the osmotic fragility procedure.

3. Erythrocyte morphology as well as reticulocytosis should be studied in relation to either or both deficiencies.

4. Vitamin E and selenium levels should be measured in plasma to correlate them with the other experimental measurements.
LITERATURE CITED


