108
86

THE EFFECT OF DIETARY ZINC LEVEL UPON THE EFFICIENCY OF VITELLOGENIN SYNTHESIS BY MALE QUAIL

by

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THE EFFECT OF DIETARY ZINC LEVEL UPON

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(abstract)

Manipulation of dietary zinc produced a different body zinc status in male Japanese quail during 3 weeks of treating period. After intramuscular injection with 8μmol/100g body weight of estradiol-17β, Japanese quail were sacrificed and vitellogenin (phosphoprotein) production was assessed in these birds by analyzing plasma for protein-bound phosphorus concentrations (PBP). Plasma PBP concentrations of estrogen-injected male quails increased from undetectable values for control birds to 0.66mg/ml for high-zinc birds and 0.42mg/ml for low-zinc birds. About 36% loss of vitellogenin synthesis was associated with the consumption of the zinc-deficient diet. Plasma zinc concentrations also increased on the estrogen injection because of the zinc-binding property of vitellogenin. The extra zinc ions in the plasma were considered to be come from the body zinc pools. Liver as well as other major tissues was believed to act as a reservoir of zinc for egg development but it was not

observed that liver was the major source of the extra zinc in the plasma.

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Table of Contents

page

Abstract ii

Acknowledgements iv

List of Tables vii

List of Figures viii

Introduction 1

Literature review 3

Biochemistry and Biological Role of Zinc 3

Zinc as a Nutrient 9

Steroid Hormone Action Mechanism 13

Vitellogenin Synthesis as a Model System 15

Zinc in Poultry Nutrition 19

Materials and Methods 21

Quail Maintenance and Husbandry 21

Laboratory Experiments 25

A. Plasma Zinc Assay 25

B. Diet and Liver Zinc Assay 25

C. Plasma Vitellogenin Extration and

Protein-bound Phosphorus Assay 27

Results 31

Establishment of Experimental Diets 31

Diet Analysis for Zinc Content 31

Growth and Diet Consumption Patterns 31

Plasma Zinc and Protein-bound Phosphorus Assay 32

Liver Zinc Assay 33

Discussion 44

Appendix 49

Statistical Analysis 49

Test for Growth 50

Test for Plasma Zinc Concentration 51

Test for Plasma PBP Concentration 52

RNA Polymerase II Activity Assay 53

Literature Cited 55

Vita 64

List of Tables

		Page
Table 1.	Zinc deficiencies in animal and human body ·······	11
Table 2.	Diet composition ·································	24
Table 3.	Zinc contents of experimental diets ··············	36
Table 4.	Individual growth pattern of quail on high zinc diet ·································	37
Table 5.	Individual growth pattern of quail on low zinc diet ··································	38
Table 6.	Daily diet consumption of quail ··················	39
Table 7.	The effect of dietary zinc on the concentration of plasma zinc and Protein-bound phosphorus (PBP) : High zinc diet ····································	40
Table 8.	The effect of dietary zinc on the concentration of plasma zinc and Protein-bound phosphorus (PBP) : Low zinc diet ·····································	41
Table 9.	Summary, Tables 7 and 8 ··························	42
Table 10.	Liver zinc assay ·································	43

List of Figures

Page

Figure 1. The role of zinc in nucleic acid metabolism and gene expression 8

Figure 2. Generalized hormone action mechanism 14

Figure 3. Flow Injection Analysis system 30

Figure 4. Weight increase as a function of diet 34

Figure 5. Diet consumption on a variation of diet 35

INTRODUCTION

Zinc was first recognized to be a requirement for normal parturition in rats when Apgar reported difficult and prolonged deliveries in female rats fed a zinc-deficient diet during gestation (1). Bunce observed that this abnormal parturition was accompanied by delays in the rate of appearance of estrogen-induced proteins in the ovaries and uterus (2). They proposed that selective gene expression controlled by estrogen action was highly dependent upon zinc availability.

The mechanism of estrogen action in regulating gene expression is well established, and there are multiple sites where zinc could affect the full expression of estrogen response. Previous studies have identified the presence of so-called "zinc fingers" in specific receptor proteins for steroid hormones including estrogen receptor protein (3,4). Hence, available zinc may influence the binding of hormone-receptor complex to DNA for the production of specific protein. Protein synthesis may also be dictated by other zinc-related events during the process including the unfolding of DNA strands (5), participation in the enzymatic reactions of zinc metalloenzymes such as RNA polymerase II (6) and aminoacyl-tRNA synthetase (7), and in polyribosome formation (8).

A current objective was to find a suitable model of estrogen induced protein synthesis and to test it at different levels of available zinc. For this purpose the focus was on the estrogen-mediated induction of the egg yolk precursor protein, vitellogenin, in the liver of male Japanese quail. Vitellogenin is a phosphoserine-rich protein which is normally produced in the liver under the regulation of estrogen and released into the blood for transport to the ovary. The protein is taken up by the oocyte and converted into egg yolk protein.

Male quail lack endogenous estrogen and do not require vitellogenin synthesis. The liver, however, possesses estrogen receptors and produces a large amount of vitellogenin in response to the appropriate dose of estrogen (9). Therefore, male quail were chosen to test the effect of zinc availability on estrogen-induced protein synthesis. Uninjected males were used to establish the basal level of zinc and vitellogenin content.

The objectives of this research included the following:

1. To use the male quail model to evaluate the role of zinc in estrogen-mediated protein synthesis.
2. To establish synthetic diets.
3. To estimate the availability of zinc in the quail.
4. To determine relationships between zinc status and vitellogenin production.
5. To evaluate the possibility of impaired estrogenic stimulation of RNA polymerase II on zinc-deficient birds.

LITERATURE REVIEW

Biochemistry and Biological Role of Zinc

Zinc is a divalent cation with an ionic radius of 0.65Å. Its two electrostatic charges and small size give it unique chemical and biological properties in contrast to other divalent metal ions such as Mg^{2+}, Ca^{2+}, Cu^{2+}, etc. Zinc differs markedly from magnesium and calcium since it has much higher affinities for electrons. It is therefore a strong Lewis acid and can bind strongly to electron donors such as thiolates and amines. Copper and nickel have also high affinities for electrons and show similar properties. However, zinc differs from copper and nickel in other respects. It does not show variable oxidation states and could be preferred in certain circumstances while copper and nickel have the potential of promoting free radical reactions because of their rather high redox activity. Zinc is present in some proteins which are known to be important for proper gene expression. The absence of redox activity of zinc prevents the production of damaging radicals that could hydrolyze RNA or DNA and possibly even the protein chain. When bound to an enzyme, zinc can form flexible coordination geometry at the active site. Thus fast reactions can be more easily carried out when zinc facilitates rapid uptake and release of molecules from its coordination spheres (10). The biochemical importance of zinc is closely connected with its association with a large number of proteins and enzymes. More than 80 zinc metalloenzymes and proteins have been discovered from diverse sources in all of the six major enzyme categories, i.e. oxidoreductase, transferase, hydrolase, lyase, isomerase and ligase. Some examples include alcohol dehydrogenase (11), carbonic anhydrase (12), carboxypeptidase (13) and alkaline phosphatase (14). The need for zinc in the catalytic sites of enzymes might be considered here. As stated above, zinc has a high charge and electron affinity and therefore it is very effective in providing chemical specificity to the protein. Enzymes without the zinc-associated centers can bind only large substrates with very high selectivity by interacting with several sites on the substrate. Examples of the substrates with which zinc reacts at the enzyme active site are small molecules like CO_2 and ethanol and terminal peptide bonds. Besides the catalytic properties of zinc metalloenzymes, structural stabilization by zinc is another function required for the proper enzyme activity (15)

An important role of zinc in gene transcription was proposed by Klug with a discovery of zinc association in a transcription factor (TFIII) from Xenopus oocytes (16). Amino acid analysis data have shown that this protein contained 9 repeating units each consisting of approximately 30 amino acid residues and two pairs of cysteines and histidines. The zinc atom is bound in a tetrahedral complex with the two pairs of cysteine and histidine residues. The linear arrangement of these finger-like loop domains seems to have a high affinity for DNA and may explain how a small protein can bind to a specific region of DNA thereby facilitating transcription. Other studies have identified the presence of similar repeating sequences in other proteins, which might be expected to form so called "zinc-fingers". Examples are steroid hormone receptor proteins including estrogen receptor protein. Instead of the TFIII consensus sequence, an alternate putative zinc binding motif has been suggested for the estrogen receptor protein which is four pairs of cysteines in the finger-like domain. Thus zinc may play a role in binding of the hormone-receptor complex to DNA for the production of the specific protein.

A functional role of zinc in gene expression has also been proposed by studies on DNA and RNA polymerases. Slater and Scrutton in 1971 were the first to discuss the presence of zinc in DNA and RNA polymerases (17,18). Since that time, a variety of DNA and RNA polymerases from both prokaryotic and eukaryotic sources have been demonstrated to possess a constant and stoichiometric amount of zinc ions. These observations were supported by their inhibition by some metal chelators such as 1,10-phenanthroline. Many zinc deficiency symptoms indicated by the impaired growth, protein synthesis and DNA and RNA metabolism were consistent with this idea.

The precise roles of the intrinsic zinc ions in all DNA and RNA polymerases have not been fully understood. Nevertheless, It has been suggested that the metal ions play at least three possible roles in the function of the polymerases : catalytic, regulatory and structural. Previous studies, carried out with E.coli and T7 phage, proposed the possible catalytic role of zinc for DNA and RNA polymerase activity (19). Zinc ion may act as a Lewis acid to interact with the oxygen atom of the 3'-OH group of the DNA primer, thereby facilitating its deprotonation and subsequent nucleophilic attack on the alpha phosphorous of the incoming nucleotide. Zinc ion may also affect the activity by coordinating with electron donating groups on the enzymes.

Furthermore, the metal ion is known to assist the reversible unwinding and rewinding of double stranded DNA, which may be required for the proper polymerase activity. Another hypothesis for a catalytic role of zinc ion involves the coordination of zinc to 2'-OH of ribose moiety or the oxygen atoms of phosphate group on the initiating nucleotide. Comparative studies of copper-containing and zinc-containing RNA polymerases from E.coli showed that these two enzymes have different efficiencies in utilizing different promoters and therefore, they suggested the regulatory role of zinc in promoter recognition and specific initiation (20).

The zinc ion may also play a structural role in DNA and RNA polymerases by stabilizing proper enzyme conformation. This structural contribution seems to be more important in eukaryotic polymerases which have larger number of subunits than prokaryotic enzymes (19). Zinc has also been recognized to be essential for polyribosome formation and for protein synthesis elongation factor which catalyzes the binding of aminoacyl-tRNA to ribosomes (8,16). The extensive role of zinc in nucleic acid metabolism is shown in Fig 1.

Further studies have demonstrated that zinc is also present in subnuclear structures and is essential for the process of cellular development and differentiation as well as for protein and nucleic acid synthesis (21). In addition, zinc, at physiological concentrations, is found in cell membranes apparently stabilizing and protecting them against peroxidative damage(22).

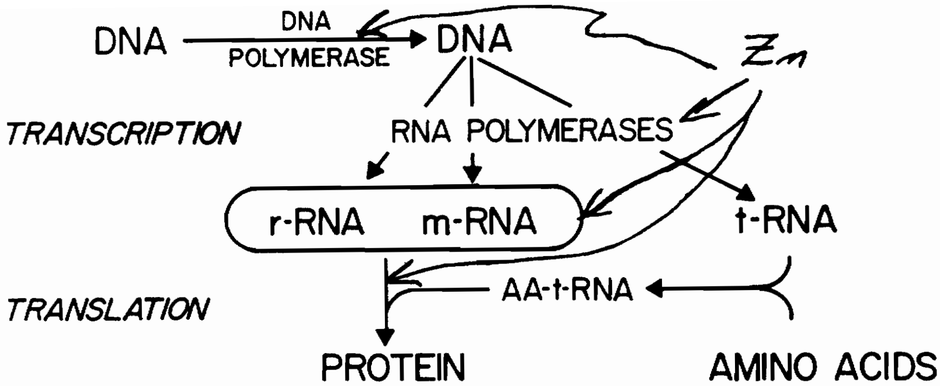

Fig 1. The role of zinc in nucleic acid metabolism and gene expression.

Zinc as a Nutrient

The presence of zinc in living organisms and its role as an essential nutrient have been recognized for almost a century. It was not until 1930's that zinc was first shown to be required for normal growth and development of animals (23). One of the major zinc deficiency symptoms is growth retardation, which was described in many animal species including rats, pigs, chicks and calves. Growth can be characterized by increase in cell size and cell number with the former being associated with protein synthesis and the latter with DNA synthesis. Previous studies suggested that impaired DNA synthesis and cell division are more responsible for the growth retardation in zinc-deficient animals (24). Furthermore, many subsequent studies, particularly on rats, suggested that the growth depression is largely the result of reduced food intake rather than of reduced zinc intake (25). Other zinc deficiency symptoms observed and experimentally produced in a variety of animal species are testicular atrophy, skin lesions and decreased size of the sex glands. All of these deficiency symptoms were observed with a decrease in food intake as the early sign of the deficient state and usually were reversed by the sufficient administration of zinc.

Little is known about the metabolic defects responsible for the symptoms, but many intensive studies have been done to elucidate the biochemical aspects of zinc deficiency. The level of zinc in living organisms governs many metabolic processes including carbohydrate, fat, protein and nucleic acid metabolism through the activities of zinc-dependent enzymes. It is, therefore, natural that changes in the activities of a number of enzymes in response to deficient zinc nutrition have been observed in various tissues. Table 1 summarizes the decreases of some enzyme activities in zinc-deficient animals along with some morphological and chemical alterations. The reduced enzyme activity may be due to the inability of low levels of zinc to maintain enzyme structure and/or catalysis. With some zinc-dependent enzymes, such as alkaline phosphatase, zinc deficiency may induce structural changes that increase the chance for degradation (11,26).

Tissues vary in their sensitivity to available zinc and some of them respond more easily to a change of zinc status than others. Serum, bones, pancreas and intestinal mucosa all respond dramatically and thus these tissues are evaluated for biochemical changes in zinc-deficient animals. Zinc metalloenzymes also have differences both in the zinc-ligand affinities and in their turnover rates in the cells of the affected tissues (27), and those with both high affinity and low turnover can still be quite active even in extreme conditions of zinc deficiency. Two examples are lactate dehydrogenase and alcohol dehydrogenase of rat liver (28,29).

TABLE 1. Zinc deficiencies in animal and human body.

Morphological changes		Reduced food intake Growth retardation Abnormal immune function Birth anomalies Testicular atrophy Parakeratosis Coarse, sparse hair Abnormal nitrogen metabolism
Chemical compositions		
	Decrease	Hemoglobin Serum protein
	Increase	Uric acid
Enzyme activity decrease		Alkaline phosphatase Alcohol dehydrogenase Pancreatic protease Leucine aminopeptidase Thymidine kinase RNA polymerase Lactate dehydrogenase Malate dehydrogenase DNA polymerase Carboxypeptidase A,B

The important role of zinc on sexual development and its relation to sex hormones has been demonstrated for various species including human beings. One obvious zinc deficiency syndrome in both animal and human males is hypogonadism which is characterized by decreased testicular weight and size, diminished number of spermatozoa and decline in the number of Leydig cells. Several studies have established that zinc-deficient rats maintain proper levels of gonadotropins but fail to maintain the population and function of the Leydig cell, the target cell for this hormone. The result is a low production and release of testosterone (30). These results have also been observed in humans as well as other animal species as the indication of zinc deficiency (31).

Zinc deficiency syndromes associated with sexual hormones and their functions have also been demonstrated in both animal and human females. Parturition was delayed and prolonged in zinc-deficient female rats (32). It was found that the decline in plasma progesterone and increase in plasma estrogen occurred normally as in the control animals (32). However, the problem may arise from impairment of two estrogen-dependent events : delayed luteolysis and inadequate development of gap junctions of myometrium through which the electrical information is transmitted for the propagation of uterine contractions (2,33). The idea that zinc is associated with the estrogen receptor proteins leads then to the possible speculation that zinc potentiates the binding of estrogen-receptor complex to chromatin and a direct function in gene expression.

Sreroid Hormone Action Mechanism

Steroids are lipids based on a carbon skeleton of four fused ring systems. They are derived from cholesterol and classified into five different groups : progestagen, glucocorticoids, mineralocorticoids, androgen and estrogen. Estrogen is mainly produced in the ovary but is also formed by aromatization of circulating androgenic precursors in a variety of other tissues including muscle, adipose tissue, hair follicles, liver and hypothalamus. Although estrogen affects nearly all vertebrate tissues to some degree, its principal effect is stimulation of growth and maturation of organs relating to the female reproductive system and the maintenance of its reproductive capacity. Estrogen initiates its action by combining with highly specific cytoplasmic receptors in target cells. A general scheme for its action is shown in Fig 2 (34,35,36).

After steroid binding and activation of the cytoplasmic receptors, the hormone-receptor complex binds to chromatin at unique sites and activates RNA polymerase. The newly formed mRNA is transported out of the nucleus to the rough endoplasmic reticulum where it participates in protein synthesis. Altered cellular function is brought about by the newly synthesized protein.

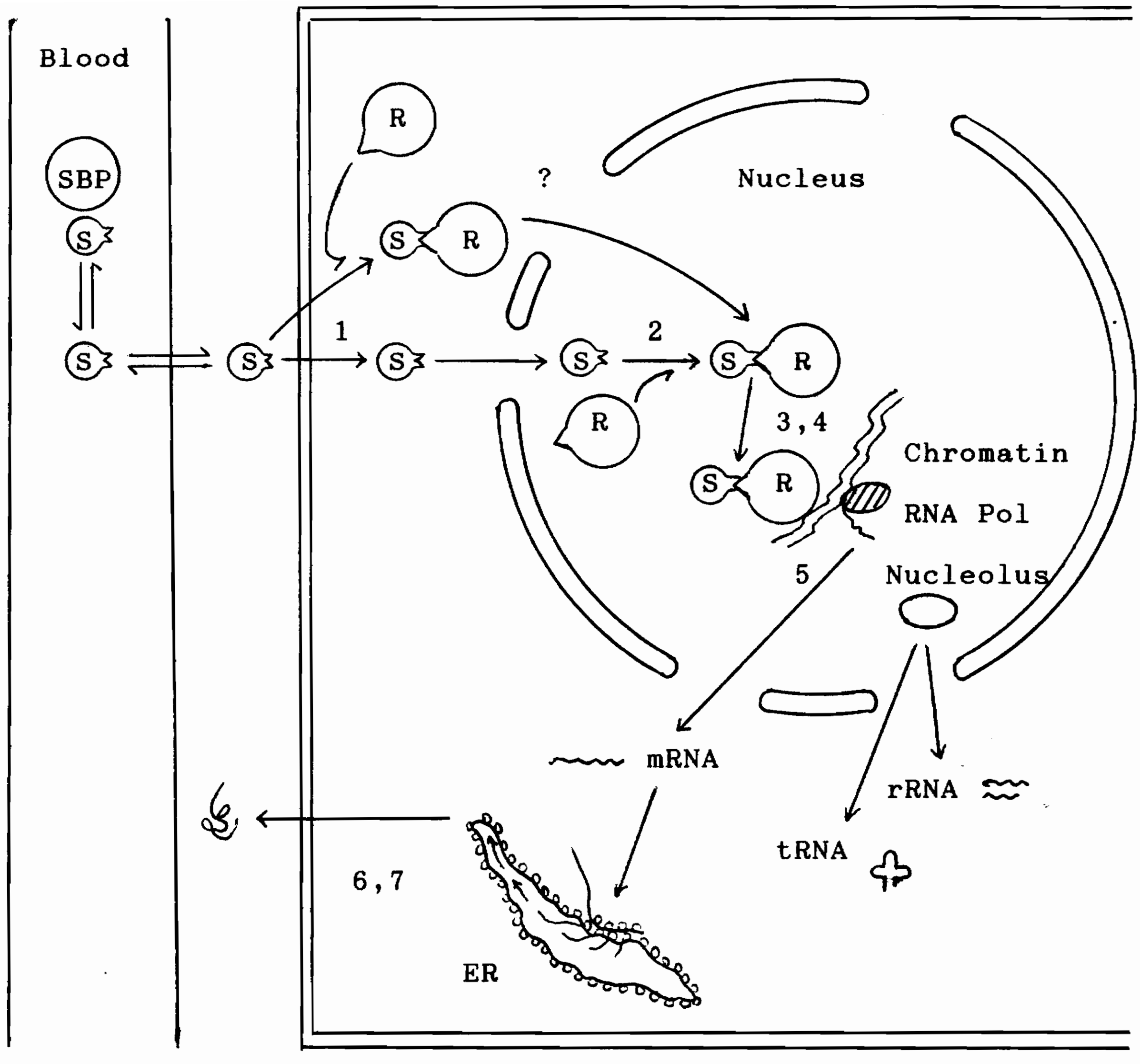

Fig 2. Generalized hormone action mechanism

1. Entry of hormone into the cell
2. Binding of hormone to specific receptors
3. Activation of the hormone-receptor complex
4. Binding of the activated hormone-receptor complex to the genome and activation of a specific gene
5. Increase in transcription and cellular mRNA levels for specific proteins
6. Increased synthesis of specific proteins
7. Altered cellular function brought about by the newly synthesized proteins

SBP:Steroid binding protein, S:Steroid, R:Recepter Protein
ER:Endoplasmic Reticulum

Although these basic ideas are well established, further study is required to fully understand steroid action. For example, binding of hormone to receptors may occur in cytosol and the hormone-receptor complex can be translocated to the nucleus for binding to chromosome (34). Recent studies, however, have demonstrated the presence of the receptors in the nucleus and suggested that hormone binds to these nuclear receptors and activates the genome directly (36). In addition to stimulating synthesis of proteins, estrogen appears to stimulate the synthesis of new receptors so that more estrogen may be bound by the cell (37).

Vitellogenin Synthesis as a Model System

The action of estrogen on formation of the egg yolk precursor protein vitellogenin in oviparous vertebrates provides a good model for studying steroid hormone action (38,39). Vitellogenin is a unique, stable protein whose synthesis is highly specific for estrogen. The induction of vitellogenin is reversible so that synthesis completely stops upon withdrawal of the hormone. Secondary induction can also be accomplished with even higher effectiveness. The induction of vitellogenin occurs in a single cell type, the hepatocyte, without cell division or cell death and results in production of large amounts of protein and mRNA which are readily isolated due to their unusual size and abundance.

Vitellogenin is a phospholipoglycoprotein with a monomer molecular weight of 210,000 to 230,000 (40). The protein is produced in the liver, released into the blood and transported to the ovary where it is selectively taken up by the oocyte being converted into two final egg yolk proteins, lipovitellin and phosvitin (39). The products of vitellogenin cleavage are incorporated into egg yolk along with other proteins. Avian vitellogenin appears to contain two molecules of phosvitin and one molecule of lipovitellin (40). The relation between the vitellogenin polypeptide and vitellogenin genes has not been fully established.

The expression of vitellogenin genes is dependent on the elevated level of circulating estrogen during the egg laying period and is coordinated with the complementary production of egg white proteins in the oviduct. Vitellogenin genes can also be induced in male avian liver by the administration of a single injection of estrogen which results in the formation and secretion into the bloodstream of a substantial amount of vitellogenin (9). Background levels of vitellogenin are nearly undetectable in uninjected male birds.

In the general model for steroid hormone action mechanism, hormone binds to specific receptors in the cytoplasm and then moves into the nucleus for the subsequent nuclear effects (Fig 2). However, it was reported that approximately equal amounts of estrogen receptors are present in both the nucleus and cytoplasm of unstimulated cells and that administration of estrogen results in the induction of both nuclear receptors and cytoplasmic receptors (41,42). These and some other observations led to the idea that the hormone freely enters the nucleus, binds to the nuclear receptors and exerts its role on gene expression.

The mechanisms for the interaction of hormone receptors with chromatin have also been extensively studied. The receptor saturation model appears to be most plausible for vitellogenin gene expression (39,43,44). In this model, the specific chromatin regulatory sites have only slightly higher affinity for estrogen-receptor complex than the other non-specific sites. Specificity, however, is obtained by the binding of the complex to chromatin and a substantial number of receptors bind with increased specificity in a cooperative fashion. This model may explain the high tendencies for hormone receptors to aggregate and the inability to find specific, high-affinity binding sites on chromatin.

The identity of the specific binding sites in chromatin has not been fully understood. However, a specific DNA sequence believed to be estrogen responsive was found in the 5' regions of vitellogenin genes in frogs and chickens (45,46,47). This 38 nucleotide sequence contained an inverted repeat (5' G-G-T-C-A-N-N-N-T-G-A-C-C 3') and an A/T-rich region. It is likely that the inverted repeat provides the primary binding site for estrogen receptor, while the A/T-rich region facilitates stable binding by allowing a conformational change in the DNA structure or by providing additional attachment sites for estrogen receptor

(48).

The induction of vitellogenesis by estrogen is characterized by the increased RNA polymerase II activity and the massive accumulation of total RNA in the liver. Vitellogenin mRNA has been purified to homogeneity using several techniques including gel electrophoresis of total cytoplasmic RNA followed by oligo(dT)-cellulose chromatography (49). Considerable enrichment for vitellogenin messenger has been obtained with this method because of the large size and stability of the mRNA and the presence of a base 3'poly(A) tail. The levels of vitellogenin mRNA produced in response to estrogen challenge have been determined either directly by hybridization using a complementary DNA probe, or indirectly by translational analysis in <u>in vitro</u> or <u>in vivo</u> systems (50,51,9).

A translational analysis <u>in vivo</u> was done by Gibbins (9). It gives some idea about estrogen-induced vitellogenesis in Japanese quail. As vitellogenin is the only phosphoprotein present in significant quantities in the plasma, its production was assessed by measuring plasma concentrations of protein-bound phosphorus. Vitellogenin was produced with a maximum level of approximately 0.83 ± 0.9 mg/ml protein-bound phosphorus in estrogen-injected males. The osmotic pressure of the plasma was affected by the increased level of the plasma protein concentration such that the total plasma volume was increased and proportionately the packed cell volume was decreased with vitellogenesis (9). Calcium content in the plasma was also increased consistent with the interaction between calcium and vitellogenin (9). A large amount of additional RNA synthesized in response to estrogen administration appears to code for proteins other than vitellogenin. This prodigious production of mRNA accounts for the observed massive proliferation of endoplasmic reticulum, Golgi bodies and mitochondria (52). Despite the previous studies, the identity of vitellogenin mRNA precursor and its post-transcriptional processing in the nucleus are still poorly understood, and more extensive studies are required to fully describe the mechanism of hormone action in vitellogenin synthesis.

Zinc in Poultry Nutrition

Zinc deficiency in poultry is characterized by retarded growth, abnormal bone development, poor feathering, dermatosis of the squamous epithelium and decreased efficiency of feed utilization (53). Deficient zinc states in poultry have been established by controlling dietary zinc content, source of protein, calcium and metal-binding agents such as phytate (54,55). A diet including plant proteins such as soybean and cereal caused zinc deficiency even when it contained normally satisfactory amounts of zinc. Animal proteins such as casein and egg white require more vigorous exclusion of zinc if a deficiency is to be produced. Phytate, the hexaphosphate ester of inositol, associated with plant proteins is believed to inhibit the effective absorption of zinc from the intestine and to be responsible for the higher zinc requirement in animals fed plant proteins. Moreover, phytate does not need to be bound to dietary proteins to exert its zinc-binding effect and the effect was directly proportional to its dietary level up to 1.0% (56). An excess of calcium aggravates zinc deficiency when it is added to diets based on plant proteins that might contain high phytate content (57). As opposed to the lower zinc availability produced by phytate and calcium, chelating agents like EDTA enhance zinc availability since they release zinc ion to the intestinal mucosa (56). There are also other compounds that aid in the absorption of zinc. For example, some amino acids such as cysteine complex with zinc and make it more available (55). Environmental factors such as water sources, rearing cages, feed containers and other equipment could also affect the zinc availability as well as the dietary sources (54).

METHODS AND MATERIALS

The model chosen for the experiment is the male Japanese quail fed diets either low or adequate in zinc. The liver produces large amounts of vitellogenin in response to estrogen. Vitellogenin is released into the plasma in milligram quantities. It is a phosphoprotein and can be readily assayed by determination of inorganic phosphate following isolation and digestion.

Quail Maintenance and Husbandry

Quail eggs were maintained in the refrigerator at the Virginia Tech poultry farm by Dr. Winston Beane. All the necessary facilities including the incubator, hatcher, heated pens and plasticized cages were located at the poultry farm. Hatch schedule was prepared and given to Mr. Orange, the supervisor of the poultry farm. About 100 eggs were incubated at a time based on the hatch schedule. After two weeks in the incubator, the eggs were transferred to the hatcher where about 60 of them hatched 4 days later. The hatched birds were placed in heated pens. Commercial starter feed (#401 or #401A) and tap water were supplied and the young birds were monitored daily. The birds achieve sexual maturity after 3-5 weeks in the heated pens. Males could be recognized by the development of scrotal sacs and spots on their chests. Only males were selected and females were sacrificed by cervical dislocation. About 20 male birds were randomly divided into two groups and placed in different plasticized cages which minimize the environmental zinc exposure. Five birds were placed in each cage. Temperature was maintained at 18-22°C and lighting was naturally regulated. One group was fed the control diet and tap water while the other group was fed the low-zinc diet and deionized water. The high-zinc and low-zinc diets were prepared at the Virginia Tech turkey farm and their components are shown in Table 2. By analysis the low-zinc diet contained less than 2ppm zinc compared to 150 ppm level in the high-zinc diet. The feed consumption for each bird group was recorded daily and the growth of each bird was monitored by measuring the weight increase every three days. Animals were injected with estrogen after consuming the experimental diets for three weeks. On the basis of a previous study (9), 8μmol of 17-β estradiol per 100g body weight was injected into the pectoral muscle (intramuscularly) using absolute ethanol as the carrier. Blood and livers were collected at suitable times of post injection by cardiac puncture and surgical means respectively. According to the previous studies, plasma vitellogenin reaches a peak at four days of post injection (9) and RNA polymerase II activity in the liver reaches a maximum at 24hours after the injection time (58). The collected blood was placed in microcentrifuge tubes and the animals were then sacrificed by cervical dislocation. The microcentrifuge tubes were centrifuged for 6min at 1400g and the supernatants (plasma) transferred to new tubes for storage at -20°C for the subsequent analysis.

TABLE 2. DIET COMPOSITION

COMPONENTS	WEIGHT (g)	PERCENT (%)
Egg white	3100	31.00
Dextrose	2204	22.04
Starch	2204	22.04
Corn oil	200	2.00
Solka floc	1000	10.00
Vitamin Premix	100	1.00
Mineral Premix	20	0.20
Calcium Phosphate	274	2.74
Ground Limestone	200	2.00
Sodium Chloride	70	0.70
Magnesium sulfate	28	0.28
Potassium Chloride	70	0.70
L-lysine HCl	30	0.30
L-methionine	100	1.00
Choline-chloride	100	1.00
Vegetable Fat	300	3.00
Biotin	0.04	
Vitamin B6	0.07	
* Zinc Sulfate	5.00	
Total	10000g (10kg)	100%

* Only in control diet

Laboratory Experiments

A. Plasma Zinc Assay

Metal free Sarstedt conical test tubes and deionized water were used throughout the procedure. The tubes were set up for the standards and plasma samples as follows.

	sample(μl)	10% TCA(μl)	H2O(μl)	dilution factor
blank		400	400	
blank			800	
sample	200	400	200	4

Zinc standard --- 1ppm, 0.5ppm, 0.25ppm

After brief mixing on the vortex, the tubes were centrifuged at 1700g for 15min to remove precipitable plasma proteins. The supernatants were analyzed for zinc using the Perkin-Elmer model 560 atomic absorption spectrophotometer.

B. Diet and Liver Zinc Assay

Metal-free crucibles and Sarstedt conical tubes were used throughout the procedure. The crucibles were dried in a drying oven at 105°C overnight. They were placed in a desiccator to cool and then weighed. About 0.6g diet or 1.0g liver were added to the crucibles and dried in a drying oven at 105°C overnight followed by the cooling and weighing. About 0.125g NBS bovine liver standard reference material (123±8 ppm zinc) was also analyzed for zinc in every batch. The dry weight of the diet, liver and NBS standard were determined by subtracting the dry weight of each crucible from the dry weight of the crucible and samples combined. The samples in the crucibles were ashed in a 440°C furnace for 24 hours. Temperature were raised by 50°C every hour, up to 440°C. After being ashed for 24 hours, the samples were kept in the furnace for additional 24 hours to cool. Ashed samples were then completely dissolved with 4ml 10% ultrex HCl and transferred to Sarstedt conical tubes. Dilutions were made properly so as to place the zinc concentration in the linearly detectable range of the instrument (1:8 for NBS standard, 1:40 for high-zinc diet, 1:10 for liver and no dilution for low-zinc diet). Zinc concentrations were measured on the atomic absorption spectrophotometer against zinc standards (1ppm, 0.5ppm, 0.25ppm). To determine ppm zinc (μg zinc/g diet or liver) the concentration was subtracted by the value for the blank, multiplied by the total dilution factor and divided by the dry weight of the sample.

C. Plasma Vitellogenin Extraction and Protein-bound Phosphorus Assay

The isolation of the non-lipid phosphorus material and the subsequent determination of phosphorus were performed essentially as described by Bergink and Chen (59,60). All glassware should be in phosphorus-free condition throughout the whole procedure. All samples and the extraction materials should be kept on ice to prevent the endogenous phosphatase activity which may cleave phosphate from vitellogenin before the extraction is completed. Aliquots of 0.1ml of the plasma samples were placed in the test tubes in duplicate. 2.0ml of 10% TCA solution was added to each sample to precipitate plasma proteins. The TCA solution was added slowly while the sample tube was being vortexed so as to minimize plasma phospholipid entrapment. The precipitates were obtained by centrifugation for 10min at 1700g and by removing the supernatants using pasteur pipettes. After another treatment of 10% TCA and centrifugation, 2ml of the following reagents were added to the precipitates separately in that order to completely remove lipids. When each reagent was added, the pellet was resuspended in the reagent by a teflon rod and vortexed.

1) Acetone
2) Ethanol/chloroform/ether (2:1:2, v:v:v)
3) Acetone

After the last washing with acetone, supernatants were removed and the precipitates were allowed to dry overnight. The dried precipitates (vitellogenin extracts) were solubilized and the inorganic phosphates were hydrolyzed from the protein by heating in 700μl 6M HCl at 105°C using a heating block. After being cooled, HCl from the samples was evaporated by air evaporation at 45°C under the hood. Dried materials in the test tubes were dissolved with 4ml deionized water and briefly vortexed. The phosphorus content of this final solution was determined by the semi-automated flow-injection system developed by Dr. Kent Stewart of this department. The inorganic phosphorus was reacted with ammonium molybdate to form ammonium phosphomolybdate which was reduced by ascorbic acid to give molybdenium blue. The blue color of the solution was measured at 660nm. Standard phosphorus samples were prepared as follows :

Phosphate conc (mg/ml)	1mg/ml phosphorus (ml)	dd H2O (ml)	Total (ml)
0	0	4.00	4.0
0.0025	0.01	3.99	4.0
0.005	0.02	3.98	4.0
0.01	0.04	3.96	4.0
0.02	0.08	3.92	4.0
0.03	0.12	3.88	4.0
0.04	0.16	3.84	4.0
0.05	0.20	3.80	4.0

The flow injection apparatus is shown in Fig 3. The standards and samples were injected through a syringe into the system in duplicates and the sample phosphorus concentrations were extrapolated from a standard curve.

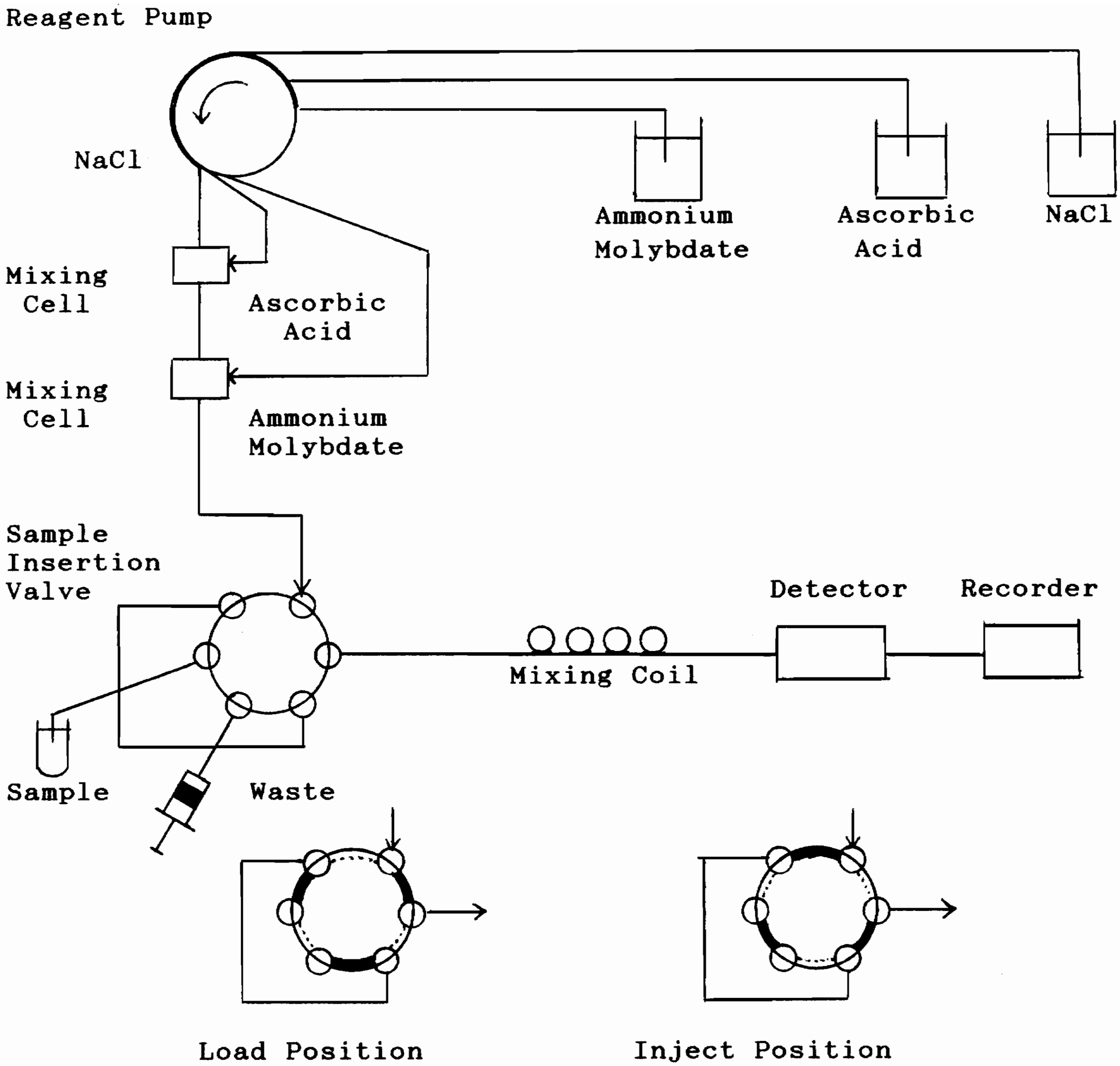

Fig 3. Flow Injection Analysis system. All the tubes and valves are flushed with the carrier solvent, NaCl, before the sample injection. Sample from a test tube is sucked by a syringe into the sample loop with the insertion valve in the load position. After filling the loop, the sample is injected onto the system by turning the valve to inject position. About 100ul of the sample is injected.

Flow rate for each reagent = 1ml/min
Flow rate for mixed solution = 3ml/min
Inside diameter, each reagent tube = 0.04mm
Inside diameter, mixed solution tube = 0.8mm
Length the mixing coil = 400cm

RESULTS

Establishment of Experimental Diets

The purpose of this experiment was to determine the components of a diet that would contribute to optimal growth of the birds. The initial objective was to determine the appropriate proportion of dextrose and corn starch in the total diet. The larger the proportion of dextrose, the greater the amount of water present in the feces. The greater concentration of corn starch makes the diet stickier and more difficult for the birds to consume. Diets with a 50:50 ratio of dextrose and corn starch gave the optimal growth pattern (Fig 4, Fig 5). The composition of the diets for all the research experiments was shown in Table 2. The diets were prepared monthly in batches of 10kg.

Analysis of Diet for Zinc Content

Two different batches of diets (both batches had exactly the same components) were prepared and analyzed. The average values are shown in Table 3. Zinc contents in the high-zinc diet were 100 times higher than those in the low-zinc diet. Therefore a significant difference in zinc content in diets was established.

Growth and Diet Consumption Patterns on the Experimental Diets

The birds were fed experimental diets (high-zinc & low-zinc) beginning at 5 weeks of age for 3 weeks. Therefore the age after three weeks of treatment was 8 weeks. The birds maintained on different dietary levels of zinc showed virtually identical growth and feed consumption patterns based on three different trials. The mean ± SD of weight increase during the 3 weeks was 20 ± 5.95g in high-zinc birds and 19 ± 4.72g in low-zinc birds. Therefore the dietary zinc content had no significant effect on the growth of the birds (Table 4,5). Feed consumption was measured daily. The measurement was performed in groups and the values were divided by the number of birds in each group. Two different groups of birds consumed similar amounts of the synthetic diets (Table 6).

Plasma Zinc and Protein-bound Phosphorus Assay

There was a difference in plasma-zinc concentration for the two uninjected control groups. Namely the plasma-zinc content for animals on low-zinc diet was only 60% of that for the other groups. There was a large increase of plasma zinc generated by estrogen stimulation of the high zinc-fed birds compared to a small increase for the low zinc-fed birds (Table 7,8,9). This phenomenon might be explained by the differences in quantity of vitellogenin synthesis stimulated by the estrogen injection. Vitellogenin produced in the liver is released to the blood-stream and the negative charges of its phosphorus portion bind positively charged metal ions such as zinc and calcium. Hence, as shown in Fig 12, about 36% loss of the protein synthesis in low-zinc birds resulted in a relatively small increase of plasma zinc.

Liver Zinc Assay

Liver-zinc concentrations were also measured for the two groups (Table 10). Zinc values in the liver of controls averaged 132 ± 15.93μg/g liver for the high-zinc birds and 79 ± 10.44μg/g liver for the low-zinc birds. The diet low in zinc caused about 60% of normal level of liver-zinc concentration. This result corresponds to the plasma zinc data which also showed 60% of normal for the low-zinc birds.

Liver zinc did not decrease significantly following hormone injection regardless of dietary zinc level. This suggests that most of the increased plasma zinc come from sites other than the liver.

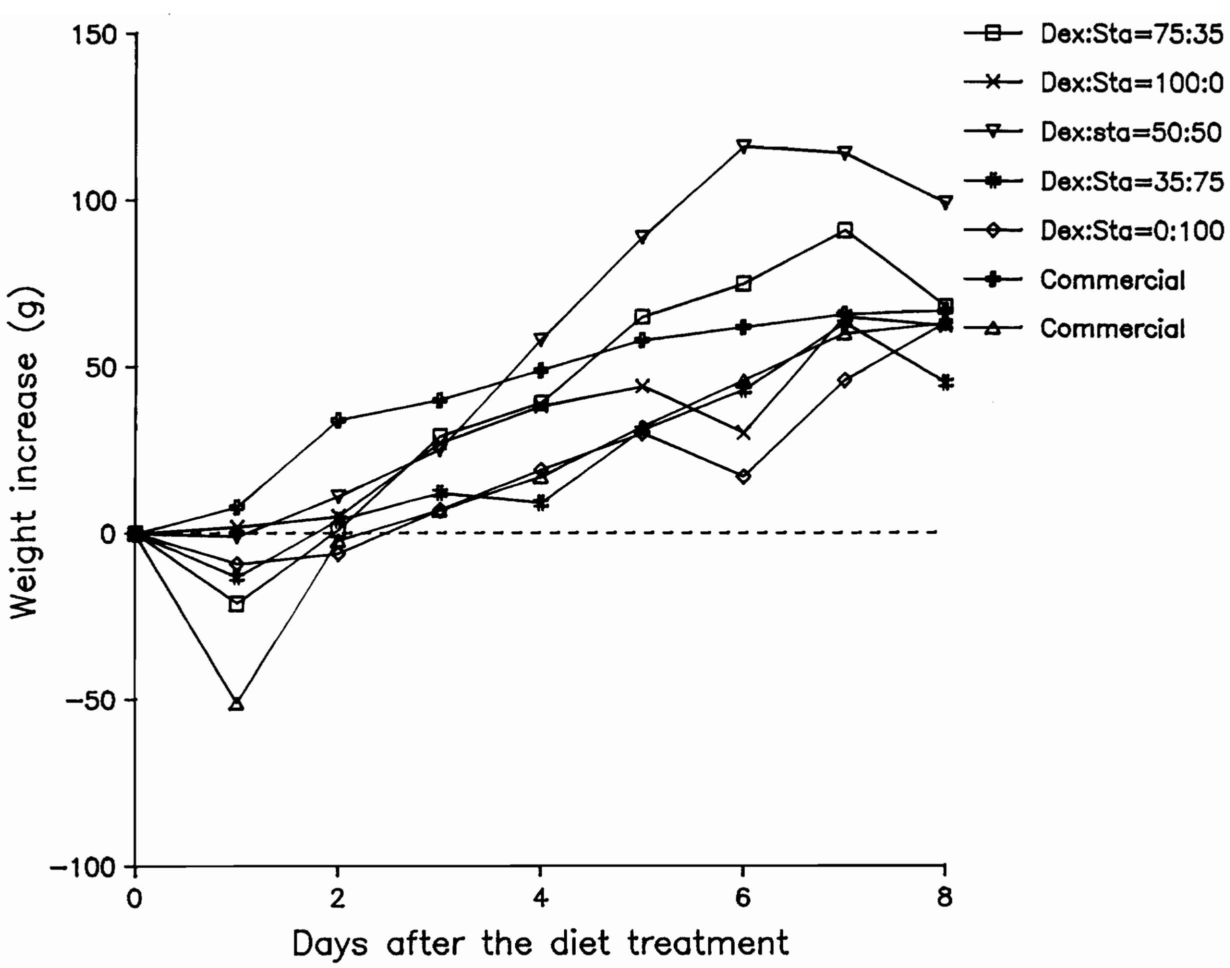

Fig 4. Weight increase as a function of diet (g/normalized group of 10). Groups of ten birds each were fed diets with deffering ratios of dextrose and corn starch. Maximum weight gain resulted from the group fed a diet containing equal parts of dextrose and corn starch, approximately 100g over an 8 day period.

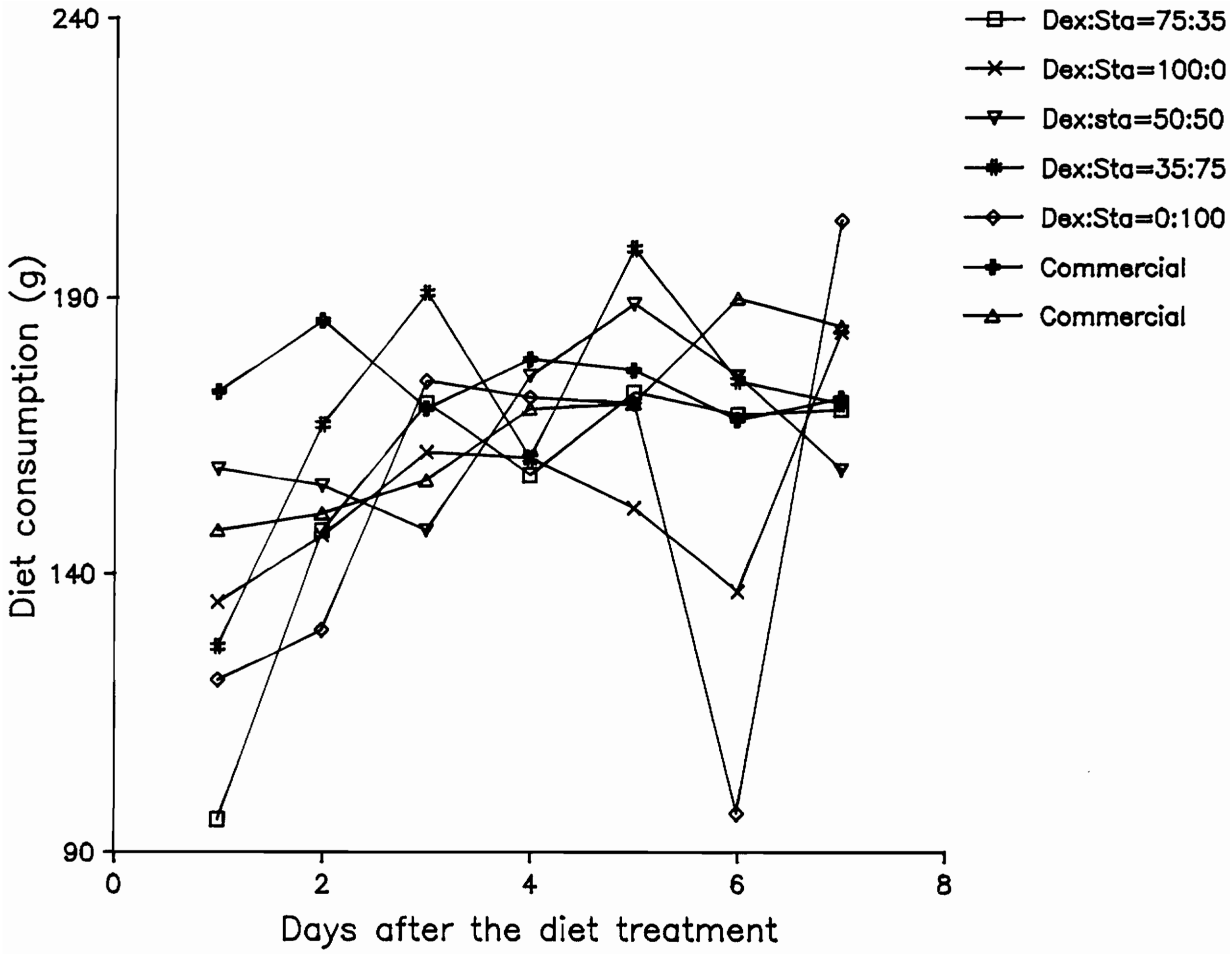

Fig 5. Diet consumption on a variation of diets (g/normalized group of 10). Feed consumption of the birds during the treatment was measured. The group on the diet with the ratio of 50:50 showed a consumption of around 165g.

Table 3. Zinc contents of experimental diets

Diet	Commercial	High zinc	Low zinc
Data (μg Zn/ g diet)	80.1	151.0	1.7
	87.9	144.2	1.3
		157.5	1.7
		151.7	1.5
		130.5	1.4
			1.4
Mean	84.0	147.0	1.5
SD	5.56	10.38	0.15

Table 4. Individual growth pattern of quail on high zinc diet

Days	0	3	6	9	12	15	18
Weight (g)	115	116	125	127	130	132	137
	121	131	135	141	142	148	151
	129	130	131	131	133	141	145
	125	127	130	126	133	145	150
	121	119	124	131	132	138	143
	121	122	134	131	131	138	137
	114	115	109	114	116	129	135
	123	122	125	128	128	137	139
	125	130	133	137	138	143	147
	116	117	120	124	126	127	128
	125	128	132	135	140	142	148
	120	121	123	127	129	129	130
	119	121	125	129	136	141	148
	123	123	127	132	133	140	142
	121	123	125	130	136	136	139
	131	133	137	141	141	139	141
	121	124	129	132	138	145	145
	117	118	120	121	122	126	130
	123	128	132	131	133	145	148
	127	130	133	131	129	133	140
	118	124	125	131	133	141	145
	121	123	127	132	133	139	138
	126	126	127	125	139	142	152
	128	133	137	137	138	144	152
Mean	122	124	128	130	133	138	142
SD	4.42	5.30	6.32	5.97	6.09	6.13	7.00

Table 5. Individual growth pattern of quail on low zinc diet

Days	0	3	6	9	12	15	18
Weight (g)	115	115	116	118	124	131	137
	120	122	124	128	131	138	143
	137	135	138	138	141	146	148
	130	138	145	140	142	143	145
	124	132	136	137	137	141	142
	112	114	122	126	128	131	136
	124	132	136	137	137	140	141
	124	125	129	133	135	137	140
	118	121	126	134	137	138	138
	131	133	134	140	142	143	149
	122	121	125	134	136	140	147
	120	120	118	122	127	130	129
	115	116	120	123	123	127	129
	119	120	123	130	131	132	133
	126	126	128	133	135	141	149
	117	120	125	130	131	135	140
	125	129	132	136	134	135	137
	119	118	121	125	127	132	139
	131	135	139	147	149	154	158
	125	129	130	136	137	142	146
	127	131	134	135	141	142	147
	117	116	121	122	129	132	138
	132	136	138	139	139	145	153
Mean	123	125	129	132	135	138	142
SD	6.35	7.58	7.76	7.14	6.51	6.35	7.20

Table 6. Daily diet consumption of quail

High zinc diet

Days	1	3	5	7	9	11	13	15	17	19	20	21
Consumption	7	18	26	26	25	33	31	29	33	32	36	34
(g/bird)	9	15	23	26	25	29	33	31	30	34	37	35
	9	19	22	29	26	30	28	35	32	36	36	33
Mean	8	17	24	27	25	31	31	32	32	34	36	34

Low zinc diet

Days	1	3	5	7	9	11	13	15	17	19	20	21
Consumption	11	21	24	28	22	30	31	30	33	31	32	33
(g/bird)	11	19	27	22	23	28	30	32	29	33	34	34
	8	15	27	26	25	30	32	34	33	36	32	35
Mean	10	18	26	25	23	29	31	32	32	33	33	34

Table 7. The effect of dietary zinc on the concentration of plasma zinc and protein-bound phosphorus (PBP).

High zinc diet

Type of stimulation	Weight	Zinc(PPM)	Pi(mg/ml)
Control	138	2.37	0
.	151	2.42	0
.	155	2.55	0
.	131	3.46	0
.	145	2.10	0
.	137	2.55	0
.	130	3.60	0
.	136	1.97	0
Injected	109	17.10	0.84
.	177	20.80	1.06
.	134	10.96	0.47
.	130	11.74	0.36
.	155	18.36	1.08
.	145	22.02	0.75
.	129		0.44
.	138	16.92	0.58
.	130	20.44	0.77
.	129	16.60	
.	146	21.52	0.70
.	114	24.28	0.64
.	150	24.88	0.62
.	118	22.48	0.74
.	148	19.30	0.65
.	124	16.20	0.51
.	109	18.14	0.63
.	130	17.98	0.61
.	159	10.98	0.66
.	124	20.30	0.76
.	145		0.81
.	118	18.24	0.41
.	149		0.46
.	151	20.30	0.71

Table 8. The effect of dietary zinc on the concentration of plasma zinc and protein-bound phosphorus (PBP).

Low zinc diet

Type of stimulation	Weight	Zinc(PPM)	Pi(mg/ml)
Control	127	1.20	0
.	115	0.94	0
.	146	2.53	0
.	134	1.52	0
.	130	1.65	0
.	134	1.74	0
.	135	1.59	0
Injected	141	5.07	
.	148	4.06	0.48
.	110	3.46	0.38
.	133	3.86	
.	137		0.54
.	104		0.46
.	138		0.25
.	156	5.04	0.51
.	145		0.25
.	143		0.48
.	143	4.66	0.48
.	117	4.48	0.43
.	115	5.26	
.	116	3.84	0.64
.	131		0.42
.	133	4.72	0.53
.	145	6.02	0.42
.	146	4.68	0.26
.	153	4.46	0.31
.	144	4.68	0.25
.	138	3.58	
.	154	4.98	
.	132	5.24	

Table 9. Summary, Tables 7 and 8.

		High zinc bird	Low zinc bird
Controls	Plasma zinc (PPM)	n=8 mean=2.63 SD=0.59	n=7 mean=1.60 SD=0.50
	PBP (mg/ml)	n=8 mean=0	n=7 mean=0
Injected	Plasma zinc data	n=21 mean=18.88 SD=4.31	n=17 mean=4.59 SD=0.67
	PBP (mg/ml)	n=23 mean=0.66 SD=0.18	n=17 mean=0.42 SD=0.12

Table 10. Liver zinc assay

Type	Status	Liver zinc (ppm)	Summery
Control	Low zinc	82	Mean = 79
		84	SD = 10.44
		92	
		66	
		71	
	High zinc	132	Mean = 133
		142	SD = 15.93
		120	
		154	
		115	
Injected	Low zinc	74	Mean = 74
		84	SD = 6.42
		74	
		66	
		73	
	High zinc	150	Mean = 130
		103	SD = 19.58
		148	
		130	
		121	

DISCUSSION

The objective of this project was to investigate the effect of available zinc on estrogen-responsive synthesis of vitellogenin in Japanese quail. The appropriate diet composition for the optimum growth and feed consumption was determined and the diets were made in a similar way for all the experiments. The high-zinc diet contained 100 times higher amount of zinc than the low-zinc diet. These two diets were believed to induce a different body zinc status.

According to previous studies, zinc deficiency induced reduced growth rate and food intake in all animal species studied. In the present experiments, however, no impaired weight gain and feed consumption has been noted during the 3 weeks of treatment of zinc-deficient diet starting at 5 weeks of age. Despite the lack of effect on growth and diet consumption, dietary zinc influenced maximum induction of vitellogenin by estrogen (Table 9). Therefore the hormone-reponsive vitellogenesis might be highly sensitive to zinc availability in the body. Differentiation of body-zinc status obtained in two bird groups during the experimental diet treatment caused a disparity in vitellogenin production ($P < 0.001$), although it was not sufficient to generate the common signs of zinc deficiency ($P > 0.2$).

About 36% loss of vitellogenin synthesis was associated with the consumption of a zinc-deficient diet. This result is consistant with recent researches suggesting the effect of zinc upon selective gene expression and protein synthesis. According to previous studies, zinc could be stabilizing the DNA double helix by interacting with both the phosphate backbone and nucleoside bases of DNA. Conformational change possibly occurring in zinc-deficient chromatin may interfere with the access of RNA polymerase II to the vitellogenin gene resulting in reduced protein synthesis. Functions of various chromatin components, namely RNA, histone and non-histone proteins, could also be defective in zinc-deficient birds because zinc normally interacts with these components.

The attempt to correlate impaired vitellogenin synthesis with diminished RNA polymerase II activity has been unsuccessful. However, RNA polymerase II is a key component of protein synthesis and it contains more than 1 mole of zinc per mole protein. Therefore it is likely that decreased vitellogenin synthesis in zinc-deficient birds is related to reduced structural and catalytic function in the enzyme.

Plasma zinc concentrations are known to be closely associated with dietary zinc intake in both animals and humans (53,54). Hence, depressed plasma-zinc concentrations have become an important standard of status assessment in zinc deficiency in laboratory animal experiments. In contrast, zinc uptake by blood cells involves many factors and therefore zinc concentrations in blood cells are not a reflection of the dietary zinc supply. The plasma-zinc content in the deficient birds was about 60% of that in the normal birds ($0.002 < P < 0.005$). Since the diet was very low in zinc, the values were expected to be less than 50% of normal based on previous studies and the decline was not as pronounced as expected (54). There might be some mechanism for the utilization of body zinc in severe zinc-deficient status in this specific species.

The estrogen injection brought about a large increase in the levels of plasma zinc in both high- and low-zinc birds (Table 9). The increase of plasma zinc might be due to the negatively charged phosphorus domains of the induced vitellogenin, which take up some zinc from the body. The increase in plasma zinc levels was markedly higher in the high-zinc birds when compared to the low-zinc birds, which might be explained by the differentiation of vitellogenin production and body zinc availability. Namely, a larger amount of the protein was produced on estrogen injection in the high-zinc birds and plenty of body zinc was available for the interaction with the phosphorus domains of the protein.

The sources of the extra zinc could be any sites of the body including bones, liver, skeletal muscle and other organs. These body-zinc pools can not only be the sources of the extra zinc in the plasma but also act as a reservoir of zinc for egg development.

Induced vitellogenin reduces the circulatory levels of Ca^{2+} in the plasma as well as those of Zn^{2+}. Decreased plasma levels of Ca^{2+} are the signal for parathroid gland to produce and release parathyroid hormone (PTH). The hormone stimulates the demineralization of bone and greately enhance the transfer of Ca^{2+} along with other metal ions to body fluid. The large increase of plasma zinc by the vitellogenin induction might be explained in relation to this hormone-controlled Ca^{2+} homeostasis.

In the present experiment, liver was analyzed for zinc to estimate how much zinc was contributed from the liver. As shown in Table 10, liver zinc contents in low-zinc birds were about 60% of that in high-zinc birds for controls, and no significantly detectable amount of zinc was depleted from the liver on estrogen injection. Since the total amount of zinc present in the liver is much larger than the total present in plasma, relatively small variations in the liver-zinc contents can have dramatic effects on plasma zinc. We can consider this aspect with an example : The total mass of blood is about 8.5% of the body weight of poultry, and plasma normally comprises 40% of the blood volume. Therefore, plasma constitutes about 3.4% of the total body weight. On the other hand, the liver accounts for about 4% of the total body weight. By calculation with the data given in Table 9,10 (plasma zinc concentration = 2.1ppm, liver zinc conc = 105.5ppm, averaged values for control birds), the total zinc contents in plasma and liver were 10.7ug and 633ug respectively for uninjected control birds based on 150g average body weight. It may be therefore concluded that contribution of the liver to the elevated levels of plasma zinc could not be confirmed by the analytical measurements.

Appendix

Statistical Analysis

Statistical procedures were performed to infer whether differences exist between the two sample groups using student t distribution (53). The hypothesis, Ho: $\mu_1=\mu_2$, can be proposed to ask whether one data group has the same mean as does the other group. The t value for testing the hypothesis concerning the difference between two means is

$$t = \frac{X_1 - X_2 - (\mu_1 - \mu_2)}{\sqrt{((Sp^2/n_1) + (Sp^2/n_2))}}$$

$$sp = \frac{SS_1 + SS_2}{V_1 + V_2}$$

where μ_1, μ_2 : mean of the population
X_1, X_2 : mean of the sample
n_1, n_2 : size of the sample
SS_1, SS_2 : sum of squares $= \Sigma(X_i - X)^2 = \Sigma X_i^2 - (\Sigma X_i)^2/n$
$V_1 + V_2$: degrees of freedom $= n_1 + n_2 - 2$

To test the hypothesis, the t value for the α significance level with V degrees of freedom, $t(\alpha,v)$, should be determined from the statistical tables ($\alpha=0.05$ for most biostatistical analysis). Ho will be rejected if $|t| \geq t(\alpha,v)$ and it will be concluded that the two samples come from populations having unequal variances. The probability (P) of making a wrong decision with the given data will also be determined.

Student t-Test for Growth

Ho : $\mu_1 = \mu_2$

Weight increase (g)	
High-zinc birds	Low-zinc birds
16	11
16	15
16	17
21	18
22	22
22	23
25	24
31	

$n_1 = 8$ $\quad$ $n_2 = 7$

$X_1 = 21 \pm 5.25$ $\quad$ $X_2 = 19 \pm 4.72$

$SS_1 = 192.88$ $\quad$ $SS_2 = 133.71$

$$Sp = 25.1223$$

$$t = 0.771$$

$$t(0.05,\ 13) = 2.160$$

$t \leq t(0.05,\ 13)$ Therefore, can not reject Ho.

Student t-Test for Plasma Zinc Concentrations

Ho : u1=u2

Plasma zinc concentration (ppm)	
High-zinc birds	Low-zinc birds
1.97	0.94
2.10	1.20
2.37	1.52
2.42	1.59
2.55	1.65
2.55	1.74
3.46	2.53
3.60	

$n_1 = 8$ $n_2 = 7$

$X_1 = 2.63 \pm 0.5940$ $X_2 = 1.60 \pm 0.4959$

$SS_1 = 2.4700$ $SS_2 = 1.4757$

$Sp = 0.3035$

$t = 3.612$

$t(0.05, 13) = 2.160$

$t \geq t(0.05,13)$ Therefore, reject Ho. $0.002 < P < 0.005$

Student t-Test for Plasma PBP Concentrations

Ho : u1=u2

Plasma PBP concentration (mg/ml)		
High-zinc birds		Low-zinc birds
0.36	0.65	0.25
0.41	0.66	0.25
0.44	0.70	0.25
0.46	0.71	0.26
0.47	0.74	0.31
0.51	0.75	0.38
0.58	0.76	0.42
0.61	0.77	0.42
0.62	0.81	0.43
0.63	0.84	0.46
0.64	1.06	0.48
	1.08	0.48
		0.48
		0.51
		0.53
		0.54
		0.64

$n_1 = 23$ $n_2 = 17$

$X_1 = 0.66 \pm 0.1841$ $X_2 = 0.42 \pm 0.1181$

$SS_1 = 0.7453$ $SS_2 = 0.2231$

$Sp = 0.0255$ $t = 4.699$ $t(0.05, 38) = 2.204$

$t \geq t(0.05, 38)$ Therefore, reject Ho. $P < 0.001$

RNA Polymerase II Activity Assay

The assay was performed by a modification of the procedure described by Glasser (61). The assay conditions had been derived from various protocols. All procedures were performed at 4°C to minimize the RNase activity. Animals were sacrificed by the cervical dislocation 24 hours post injection of 17-β estradiol. Control animals were also sacrificed at 24 hours after injection with the ethanol. Fresh livers were removed by surgery, blotted dry and quickly weighed. The livers were then placed into 10 volumes of cold homogenization solution which contained 0.25M sucrose in TKM buffer : 0.01M Tris-HCl, 5mM MgCl2, 25mM KCl, PH 7.5. The livers in the solution were minced with scissors and then homogenized with 4 strokes in a homogenizer (Sears, Model 315.11970). The homogenates were mixed with two volumes of the same homogenization solution and filtered through cheesecloth into the ultracentrifuge tubes. Two volumes of 1.6M sucrose solution were underlaid into the bottom of the tubes. Samples were then centrifuged for 1 hour at 40000g in a ultracentrifuge to pellet the nuclei. The nuclear pellet was suspended in a small volume of 25% glycerol solution in TKM buffer (a half volume per original g wet weight liver) and briefly homogenized by hand. Enzyme solutions (40μl) were incubated at 20°C for 10min in the reaction mixture (250μl final volume), which contained 10μmol of tris-HCl, 0.4μmol of 2-mercaptoethanol, 0.1μmol of sodium phosphate buffer, 0.16μmol each of ATP, GTP and

CTP, 0.05μmol of UTP, 2.45μCi of 3H-UTP (49Ci/mmol), 0.4μmol of MnCl2, 50μmol of ammonium sulfate, and 0.5 μmol of MgCl2. The reactions were initiated by the addition of the enzyme and achieved both in the presence and absence of 0.2 μg α-amanitin. The α-amanitin is a specific inhibitor of RNA polymerase II. The reactions were terminated by adding 1ml of 10% cold TCA and centrifuged at 1700g for 2min. The acid-insoluble precipitates were washed with 2ml of cold 5% TCA-1% $Na_4P_2O_7$ and centrifuged again at the same speed. The precipitates were then collected with vacuum on nitrocellulose filters (0.45μm in pore size and 25mm in diameter), washed with water and toluene and dried for an hour. The filters were counted for radioactivity in 5ml of fluor using a Beckman liquid scintillation counter. Counting efficiency determined by external standard channel ratio method was about 35%. The average CPM for the blank reaction mixtures was subtracted from that of sample assay mixtures. RNA polymerase II activity was calculated by subtracting the incorporated 3H-UMP in the presence of α-amanitin from that in the absence of α-amanitin and reported as pmol of 3H-UMP/mg of DNA.

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