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Effect of Zinc Nutriture and Contraceptive Steroid Injection on Body Composition of Young Female Rats

Sang Ai Hahn
Utah State University

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EFFECT OF ZINC NUTRITUDE AND CONTRACEPTIVE STEROID INJECTION ON BODY COMPOSITION OF YOUNG FEMALE RATS

by

Sang Ai Hahn

A thesis submitted in partial fulfillment of the requirement for the degree of

MASTER OF SCIENCE in

NUTRITION AND FOOD SCIENCE

UTAH STATE UNIVERSITY
Logan, Utah

1975
ACKNOWLEDGMENTS

The author would like to express appreciation to Dr. Deloy G. Hendricks, Dr. LeGrande Ellis, and Dr. Arthur W. Mahoney for their teachings, which became the very beginning and the result of this study. I also thank to all of the individuals who helped in accomplishing this work, a special thank you to Miss Margaret Chamber for technical help and to Dr. Deloy G. Hendricks for financial aid for this experiment.

I dedicate this dissertation to my mother, Mrs. Tong Ki Chang, who has been supporting me with confidence and patience in my work and whom I respect the most.

Sang Ai Hahn
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ABSTRACT

Effect of Zinc Nutriture and Contraceptive Steroid Injection on Body Composition of Young Female Rats

by

Sang Ai Hahn

Utah State University, 1975

Major Professor: Dr. Deloy G. Hendricks
Department: Nutrition and Food Science

Forty-nine female rats initially weighing about 95 grams were used in this experiment to determine the effect of contraceptive hormones on body composition in zinc deficient and zinc supplemented rats. Four treatment groups were: 2 ppm of dietary zinc with daily contraceptive steroid injections, or with placebo injection, and 140 ppm of dietary zinc with contraceptive steroid injection or with placebo. The daily injected steroids consisted of 0.1μg of Ethynyl Estradiol and 0.1mg of Medroxyprogesterone in 0.1ml of corn oil. Non-hormone treated groups received 0.1ml of plain corn oil. All injections were administered into the Gluteus Maximus muscle. Food intake, weight gain and serum zinc level were determined weekly. After eight weeks of experimental treatment, animals were killed by decapitation.

Livers, adrenals and ovaries were removed, weighed and frozen until cholesterol determinations could be made. The remaining carcass minus the intestinal tract was autoclaved and homogenized. Carcass moisture, ash, total lipid and cholesterol ester levels were determined. Carcass zinc and calcium levels were also determined.
The results indicate that zinc deficiency causes a marked loss of appetite with a reduced growth rate and a reduced feed efficiency in the growing female rats. Serum zinc level was also reduced. Rats fed the zinc deficient diet had smaller ovaries than the controls, but their adrenal weights were unaffected. Total cholesterol content of the adrenals was, however, increased. Zinc deficient diet caused a significant elevation of carcass cholesterol esters and carcass water content, and tended to decrease liver cholesterol level. Carcass ash concentration was increased in zinc deficient rats on the basis of fat free samples.

Hormone injection caused a slight reduction in growth rate, irrespective of zinc intake, although it slightly improved appetite in the zinc deficient rats, and which caused a slight further decrease in serum zinc level. The relative weight of adrenals and ovaries were significantly decreased by hormone administration, having markedly increased total cholesterol levels. Hormone treatment tended to increase carcass cholesterol ester and liver cholesterol levels. In zinc supplemented rats, carcass total lipids was reduced, while carcass lipid was elevated in zinc deficient rats by hormone injection. Hormone treatment elevated carcass ash content with an increase in its calcium and zinc content which was statistically significant. This increase indicates the anabolic effect of contraceptive steroids on mineral in general.
INTRODUCTION

It has been found that zinc is contained in most food stuffs in appreciable amounts. Since zinc is needed in both plants and animals for growth, most vegetable and animal organs are a good source of zinc for humans, particularly those which are high in protein. Which could explain why, only recently, the investigation of the importance of zinc in human nutrition began, even though zinc is such an important mineral in animal growth.

An unbalanced diet or a diet consisting of too little animal products, with many vegetables or a consumption of large amounts of antagonistic nutrients, particularly minerals such as calcium, copper, etc., are known to be factors in the development of zinc deficiency.

As a human example, dwarfed boys from Iran and Egypt, were reported to be zinc deficient (Prasad et al., 1969). Their diet was found to consist of mostly legumes and whole grains or other vegetables. This was reported almost a century after the announcement of the essential role of zinc on the growth of Aspergillus Niger by Roulin in 1869 (Prasad et al., 1967).

Hypogonadism, dwarfism, iron deficiency anemia, etc., were found in Iranian adult males who were identified as zinc deficient. In addition to these findings, much research has been done using experimentally zinc deficient animals to investigate the role of zinc in living organisms. For example, zinc was found to be a constituent of some metaloenzymes such as liver and yeast alcohol dehydrogenase, carbonic anhydrase, etc. (Prasad et al., 1967, Kfoury et al., 1969).
These findings suggest that zinc is necessary for the formation and activity of zinc requiring enzymes. Kfoury et al. (1969) said that, "zinc is required for maintaining the active states of enzymes and/or is required as a cofactor of certain enzymes."

It has also been demonstrated that zinc plays an essential role in the synthesis of nucleic acids and DNA and proteins (Terhune et. al., 1972; Hsu and Chesters, 1968). Impaired incorporation of labeled amino acids into DNA and protein resulted when rats were fed zinc deficient diets.

Zinc deficient diets effect carbohydrate metabolism. Zinc deficient animals show an abnormal glucose tolerance test (Hendricks and Mahoney, 1972; Quarterman, 1972; Huber and Gershoff, 1973) and an increased fasting blood glucose level. Boquist and Lernmark (1969) also stated that the pancreas of chinese hamsters fed a zinc deficient diets, showed a reduced granulation of the beta-cells, which may indicate a decreased insulin store. This change in insulin physiology may be responsible for the abnormal carbohydrate metabolism in zinc deficient animals.

Unlike the accessory sex organs of males, the female genital tract does not show such an unusual high zinc content. However, a zinc deficiency was produced in female rats as same as in male rats by zinc deficient diet (Swenerton and Hurley, 1968). An abnormal estrous cycle has been observed in young female rats fed a zinc deficient diet (Apgar, 1970). They failed to breed after a prolonged zinc deficient dietary treatment. In addition, it has been shown that a fluctuation of serum zinc level is caused by pregnancy or by
contraceptive therapy (Halsted and Smith, Jr., 1970). Also zinc
deficient diets result in growth inhibition, skin lesions, abnormal
-glucose tolerance test, etc., in female rats.

In addition to the lowered serum zinc level, animals treated
with contraceptive steroids show significant metabolic changes. For
instance, an increased fasting blood glucose and serum lipids, etc.,
has been reported (Paul, 1973). It was mentioned that oral con-
traceptive users and pregnant women are more susceptible to a
diabetic like condition (Kalkhoff et al., 1972).

Because of the directly linked relationship between metabolism
of lipids and carbohydrate, any changes in lipid metabolism could be
produced secondarily to other alterations known to affect it. For
instance, the changes in carbohydrate metabolism caused by zinc
deficient diet could be one of the factors to alter lipid metabolism.

Investigations on the tissue lipid metabolism in zinc deficient
animals have not been carried out extensively in the past.

The present study was designed to obtain information on body
composition especially, total body and liver lipid levels and their
cholesterol contents and changes in the weights and cholesterol levels
of adrenals and ovaries, which were caused by zinc deficient diet
and contraceptive steroid administration. These findings could aid
in the understanding of mechanisms involved in the regulatory effects
of zinc and/or contraceptive steroid hormones on lipid metabolism.
Zinc in General

The significance of zinc on growth was first discussed in reference to the growth of Aspergillus Niger by Roulin (Prasad, 1969) a hundred years ago. Since this initial interest, little research concerning zinc nutriture was conducted until the last few decades. In 1934, the necessity of zinc for growth of rats by Todd et al., was reported for the first time indicating an importance of zinc in animals. Following this research, extensive works on experimental zinc deficient animals has been studied, such as O'Dell's (1969) study on zinc deficient birds, the production of parakeratosis in zinc deficient pigs, growth inhibition in rats, chickens, etc. (Prasad, 1969), the alteration of DNA, RNA and protein metabolism in various tissues of zinc deficient animals (Hsu et al., 1970; Griffith et al., 1972; Williams and Chester, 1970), zinc deficiency and adrenal activity by Quarterman (1972).

Of great interest was the report in 1961, which described sexually retarded dwarfed adolescent boys from socio-economically deprived classes in Iran and Egypt, to be zinc deficient (Prasad et al., 1973). This re-emphasized the importance of zinc in animals, and this clearly indicated zinc to be significant in human nutrition.

Since zinc has been found to be necessary especially on growth, growing animals are used in experiments for the study of zinc. The
initial reports of zinc deficiency have indicated zinc to be a part of several metalloenzymes, to influence protein synthesis, to influence appetite, and the metabolism to be altered by several disease states and by certain drugs. For instance, low hair zinc levels were discovered in preschool children with poor growth and anorexia by Hambidge et al. (1971). Not only in humans but also in other experimental animals, a decreased diet consumption is a dominating and initial sign of zinc deficiency. Chesters and Quarterman (1970) suggested that one or more metabolites might accumulate in a body tissue, which interfere with appetite. They reported a quick decrease in diet consumption with a characteristic change in food intake pattern. In rats, total plasma protein was decreased significantly as compared with that of controls (Tao and Hurley, 1971). Griffith and Alexander (1972) stated that an altered amino acid metabolism in zinc deficient rats may be responsible for this change in appetite. Hambidge et al. (1971) reported an impaired taste acuity by school children who had low hair zinc level with anorexia. However, how and/or why alterations in plasma protein level can be responsible for changes in food intake is not known.

It has become clear that the roles of most trace elements, are associated with proteins such as enzymes. Several metalloenzymes containing zinc as a part of the molecule have been discovered: carboxypeptidase from bovine and porcine pancreases, cleaves a carboxy terminal peptide bond, alcohol dehydrogenase, which is found in the human and beef livers, oxidises ethanol or reduces acetaldehyde, carbonic anhydrase in human erythrocytes, metallothionein in horse kidney and similar protein in human kidney, etc. Related studies have been
conducted which show the necessity of the metal in specific activities of the enzymes. For instance, chelation of zinc in the zinc metalo-enzymes, resulted an inhibition of the enzyme activities. Zinc was stated to stabilize the protein structure of the enzymes containing zinc in them, preventing their rapid degradation (Prasad and Vallee, 1969). Generally, the activities of the zinc metaloenzymes were believed to be reduced by depriving zinc from the experimental animals or from the tissue preparations (Hsu, 1966). Stirn, Elvehjem and Hart (1935) reported that the reduction of carboxypeptidase might cause an inhibited proteolysis, particularly, in the intestinal tract, which in turn, might result in poor food utilization. Only pancreatic carboxypeptidase A, while no significant change in carboxypeptidase B, was reduced in zinc deficient rats (Hsu et. al., 1966). Also, a lower activity of intestinal phosphatase was shown in zinc deficient animals compared to that in zinc supplemented ones (Kfoury et. al., 1969). However, several liver enzymes such as lactic and alcohol dehydrogenases (Hsu et. al., 1966) glutamic dehydrogenase (Huber and Gershoff, 1973) were not influenced by zinc deficient diet.

Zinc, also, is found to be an essential component of several dehydrogenases involved in carbohydrate metabolism; for example, isocitrate dehydrogenase, an enzyme in the tricarboxylic acid cycle. Theuer and Hoekstra (1964) stated that in microrganisms, zinc deficient treatment resulted in a defective carbohydrate metabolism.

The pancreas contains a appreciable amount of zinc, especially in the Beta Cells. Furthermore, it has been mentioned that the zinc in the Beta Cells decreased due to the secretion of insulin (Boquist and
In 1934, Scott showed the role of zinc in the crystalization of insulin, and suggested that zinc prolongs the physiological action of insulin.

Zinc deficient diets cause a reduction in zinc content of the pancreas which is accompanied with abnormal signs (Macapinlac et al., 1966). Such signs, which are believed to be mediated via altered insulin physiology, are an abnormal glucose tolerance curve (Hendricks and Mahoney, 1973; Quarterman, 1971) and elevated fasting blood glucose level. These might indicate an altered carbohydrate metabolism caused by zinc deficiency. However, the role of zinc in the Beta Cells of pancreas associated with insulin physiology, is not clearly understood.

**Zinc and Proteins**

The question exists, as to what is the basic effect of zinc that its deficiency can cause a failure in growth and several pathologic manifestations such as impaired wound healing, skin lesions, etc. Although the role of zinc in nucleic acid and protein synthesis is poorly explained, it has been assumed for the fundamental reasons for these symptoms stated above. For instance, Hsu and Anthoney (1969) reported a reduced incorporation of labeled amino acids into protein in the liver and skin preparations; and DNA formation by kidney medium, was, also, reported to be interfered by zinc ion removal (Fujika and Lieberman, 1964). As a matter of fact, Williams and Chesters (1970) found a decreased activity of nuclear DNA dependent RNA polymerase in the liver of zinc deficient rats. Theuer and Hoekstra (1964) reported a considerably increased oxidation of intravenously administered labeled leucine and lysine by zinc deficient rats in comparison with that by zinc supplemented rats. Tao and Hurley (1971) suggested that protein
breakdown was to be increased and others said that protein synthesis was defective in zinc deficient rats. Other disturbances in amino acid metabolism, such as a change in sulfur containing amino acids and an increase in plasma free amino acids, were observed by Griffith and Alexander (1972). Connective tissues are high in hydroxyproline. An increased urinary excretion of hydroxyproline occurs in zinc deficient rats. It has been postulated that may result in the retarded growth rate of the zinc deficient animals (Griffith and Alexander, 1972).

Relationship between zinc and Adrenal Steroid Hormones

The relationship between adrenal steroids and zinc has been studied. The primary role of the steroid hormones is suggested to be their involvement in specific protein synthesis, which is, generally, understood as a mechanism of the action of the hormones. Cox and Ruckenstein (1971) mentioned that zinc accumulation in cell cultures is required for protein synthesis, and adrenal steroid hormones induced this accumulation. Flynn et al. (1972) reported a decreased synthesis of steroid hormones resulted when zinc was chelated from adrenal homogenates. In the zinc deficient Egyptian dwarfed boys, the adrenal response to exogenous ACTH to produce adrenal corticosteroid, appeared to be lower than that of normal's. Those dwarfs, also, showed decreased urinary pituitary gonadotrophins and 17-ketosteroids (Sandstead et al., 1967). Prasad and Oberleas (1970) also stated a decreased pituitary adrenocorticotropic hormone reserve in zinc deficient subjects. Whether or not this could be responsible
for the reduced steroids synthesis in those zinc deficient subjects is not clear, however, a decreased zinc content in adrenals was reported in zinc deficient rats (Miller, 1969).

Recently, attention has been focused on hormonal regulation of tissue zinc metabolism. Adrenalectomy of rats results in a decreased serum zinc level and an increased labeled zinc accumulation by the livers and a significantly increased liver zinc content (Dorn and Gunther, 1970). Miller (1969) showed an increase in labeled zinc accumulation in the livers of zinc deficient rats. Adrenalectomy results in failure to respond to adrenocorticotrophin stimulation to raise circulating adrenal steroids, which, in turn, will continuously stimulate ACTH release into the circulation. Similarly, Macapinlac et. al. (1966) stated that the adrenals of the zinc deficient animals were hyperfunctional. Presumably, it is because the zinc deficient diet might cause a nutritional stress, and this stress of zinc deficiency may produce hyperfunctional adrenals medicated by an enhanced ACTH stimulation. Quarterman (1972) reported that hypersensitive adrenals resulted from feeding rats a zinc deficient diet. The increased adrenal cholesterol and ascorbic acid levels found by Quarterman may indicate an insufficient stimulation of ACTH or surpressed activity of cholesterol esterase or an inhibited metabolic pathway of steroid hormone synthesis from cholesterol by some unknown mechanism. This could be similar to the statement of Apgar's (1972) which is that the adrenals of the zinc deficient pregnant rats might be enlarged but be non-functional.

McBean et. al. (1971) treated female rats with contraceptive steroids, and found a decreased serum zinc level and an increased zinc
accumulation in their livers. The administration of contraceptive steroids results in an increase in the circulating adrenal steroids and ovarian steroids, which, in turn, suppress syntheses of these steroids by adrenals and ovaries.

From these findings above, it may be summarized that zinc deficiency causes an elevation of circulating ACTH, but the adrenals in zinc deficient animals may not respond efficiently to meet the increased ACTH stimulation. If this supposition is reasonable, the state of steroids in the blood of the zinc deficient animals becomes similar to that of adrenalectomized rats. Both cases had decreased serum zinc levels. Therefore, a properly functioning adrenal cortex may be related to maintaining proper body zinc levels (Flynn et al., 1973).

The levels of tissue copper and zinc and liver cholesterol synthesis

It is a common occurrence that female sex hormone administration and pregnancy cause a sharp increase in ceruloplasmin. Usually an increase in serum copper level accompanies a decrease in serum zinc level. Decreased serum zinc levels in zinc deficient animals (Recka, 1970) and in contraceptive steroid users (Schenker et al., 1971) is accompanied by an increase in serum copper levels. And a toxic level of dietary zinc resulted in an increased zinc content and decreased copper content in the livers (Cox and Ruckenstein, 1971).

Hypophysectomy and adrenalectomy caused an elevation of serum copper while they reduced serum zinc levels (Evans and Wiederanders, 1967). In summary, it seems that factors which cause a lower tissue zinc level also produce an elevation of tissue copper level. Thus,
an alteration in the body zinc distribution induced by various reasons, might cause a change of the ratio of zinc to copper in the tissues, for instance, in the livers.

Of interest in regards to copper and zinc levels of livers in relation to hepatic cholesterogenesis was developed in the recent report by Klevay (1973). This report says that an increase in the ratio of zinc to copper in the livers, particularly, by increasing amount of dietary zinc, favors cholesterol synthesis by livers, leading to an elevation of plasma cholesterol level. Unfortunately, there are not many papers available showing tissue copper and zinc contents in experimental animals concerning hepatic cholesterol synthesis.

Evans et. al. (1970) stated that estrogen may induce the synthesis of ceruloplasmin RNA templates resulting in increased serum ceruloplasmin. But the copper content in the livers of Estradiol treated rats, appeared to be reduced. A decreased liver copper concentration in estrogen treated female rats, was reported by Kritchevesky et. al. (1963). Zinc level in the livers of estrogen treated rats was not examined in the above two reports. McBean et. al. (1971) reported that contraceptive treatment of female rats resulted in an increase in accumulation of labeled zinc in the livers. Kritchevsky et. al. (1963) reported a higher cholesterol content in the livers of estrogen treated female rats compared to that in the controls'. Perhaps, the contraceptive steroids treatment of female rats could cause elevated zinc accumulation with decreased copper content in their livers, and where more cholesterol was contained than controls.

In zinc deficient animals, except for a slightly elevated serum cholesterol level, nothing consistent has been reported about the
cholesterol levels and tissue mineral levels. Dalvi (1971) reported no significant difference in liver zinc content between zinc deficient and supplemented rats. Prasad, Oberleas, Wolf, and Horwitz (1967) showed a tendency for elevated liver copper i. e. animals fed a zinc deficient diet.

**Lipid Metabolism**

**Introduction**

Tissues such as the liver and adipose tissue, are the main sites for triglyceride synthesis. Lipid metabolism is primarily regulated by the hormone insulin, so is carbohydrate metabolism. These two metabolic processes are directly related to each other.

Pyruvate from carbohydrate metabolism is converted to acetyl CoA which combines with glycerol-3-phosphate to convert phosphatidic acid and to diglycerides. This is the common path for the synthesis of triglycerides and phospholipids. The enzyme in the endoplasmic reticular condenses three molecules of acetyl CoA to make Beta-Hydroxy-Methyl glutaryl CoA and which is converted to cholesterol via mevalonic acid.

For instance, in fasting or in diabetic animals the Beta-Hydroxy-Methyl glutaryl CoA is cleaved to acetoacetate and acetyl CoA instead of going to mevalonic acid. The acetyl CoA in the cells causes a diminished conversion of pyruvate to acetyl CoA. This accumulation may be produced by enhanced oxidation of long chain fatty acids or oxidative decarboxylation of pyruvate. In short, it can be induced by abnormal nutritional and hormonal state. The suppressed conversion of acetyl CoA from pyruvate, in turn, causes a diminution of the insulin
activity in glucose transport into the cells to decrease the rate of glucose-6-phosphate formation. For example, in diabetics, the oxidation of glucose-6-phosphate appears to be low, which results in reduced fatty acid synthesis.

Increased blood glucose and free fatty acids seen in the blood of experimental animals such as the rats fed zinc deficient diet or animals on oral contraceptive steroids treatment, may be caused by an alteration of carbohydrate and/or lipid metabolism.

**Effects of steroids on triglyceride synthesis**

Gracia et. al. (1973) stated that oral contraceptive treatment decreased the tissue enzyme lipoprotein lipase activity. The enzyme is responsible for breakdown of triglycerides. So the lowered activity may cause an elevation of circulating triglycerides but which is not available for tissue (Vaughan and Stanley, 1963). Also the enzyme of diglyceride acyltransferase, which stimulates liver triglyceride synthesis from diglycerides, has been reported to be decreased (Young, 1972). Through these findings, it seem that rats treated with estradiol would have a reduced weight compared to the controls.

The contraceptive steroid hormones treatment of women has been reported to cause elevated blood insulin level, which is an anabolic hormone on carbohydrate and lipid synthesis. However, another report also stated the increased insulin is inactive (Spellacy, 1973). Oral contraceptives causes increased levels of Xanthuronic acid due to a depression of pyridoxine. Xanthuronic acid in turn inhibits insulin action. These statements indicate that the effects of contraceptive steroids on insulin activity is rather negative.
Effects of steroid hormones on cholesterol metabolism

Most tissues can synthesize cholesterol but only liver can catalize or inactivate steroids. The cholesterol is contained throughout the whole body. The degree of turnover varies with tissues; such as that cholesterol in the brain is not exchangeable with blood cholesterol. Also, the percentage of ester cholesterol varies from tissue to tissue.

It has been shown that hepatic cholesterol synthesis is under feedback control by dietary cholesterol, fasting, etc. The regulatory effect of steroids also has been emphasized.

Acetyl CoA, serving as a beginning precursor, is condensed into hydroxy-methyl-glutaryl CoA (HMG CoA), which is then converted to mevalonic acid by hydroxy-methyl-glutaryl CoA reductase. So either inhibition or stimulation of this enzyme will likely influence tissue cholesterol synthesis. For example, Makherjee and Bhose (1968) reported a decreased activity of HMG CoA reductase when 17-Beta-Estradiol was administered to male rats. And this steroid has been shown to lower serum cholesterol (Lee, 1971). This might be induced by the suppressed synthesis via reduced synthetic enzyme activity.

However, the effects of steroid on hepatic cholesterolgenesis presented in literature, is not consistent. The effects vary greatly from species to species. For instance, pregnant or estrogen treated rabbits are reported to have an increased serum cholesterol while women and rats using oral contraceptives had a slightly decreased (Zilversmit, 1972), or unaffected serum level (Paul, 1973). Estrogen treatment may not cause appreciable changes in serum levels, but still cause the liver cholesterol content to be elevated.
Zilversmit (1972) said that estrogen administration caused a shortened biological half life of plasma cholesterol, which, in turn, led to accelerated cholesterol synthesis in the liver. Similarly, the rate of incorporation of labeled acetate into cholesterol in the liver was higher in females than in males (Patel, 1969).

It seems to follow that female sex hormones stimulate hepatocellular sterogenesis from the above statement.

In contrast, the inhibited HMG CoA reductase by estrogen treatment, theoretically, decreases the cholesterol synthesis from HMB CoA in the liver (Mukerjee and Bhose, 1968). Estrogen treatment of liver preparations from males and females, caused a diminished cholesterol oxidation (Kritchevsky et al., 1963). Therefore, the liver would have a higher cholesterol concentration. Kritchevsky et al. (1963) showed that the livers of estrogen treated female rats, contained slightly more cholesterol than those of controls.

Although, the mechanism of estrogen action in liver cholesterol synthesis is far from clear, at least, it is clear that the treatment is responsible for the increase in liver cholesterol level.

Also, lipoproteins and other phospholipids are shown to be elevated by estrogen administration. Plasma lipoprotein synthesis by the liver influences tissue cholesterol distribution. Plasma high-density-lipoprotein containing large amount of lecithin, is said to be increased by estrogen treatment. This lipoprotein functions as transportation of cholesterol from one tissue to the other.

Besides the effects of estrogens, Hickman et al. (1972) reported the role of corticosterone on the hepatic cholesterol synthesis.
The paper indicated that corticosterone had an inhibitory influence on the synthesis and, expectedly, adrenalectomy caused a significant rise in the synthesis.

Very recently, Klevary (1973) announced one of the conditions which favor hepatic cholesterogenesis. According to Klavey, the increase of the ratio of the zinc to copper in the liver stimulated cholesterol synthesis.

Since the effect of mineral composition of the liver concerned with cholesterol synthesis, is a recent subject, there are not enough reports to support the result of Klavey's.

**Esterification of cholesterol and tissue ester cholesterol level**

The liver is stated to be responsible for the appearance of ester cholesterols in the tissues such as adrenals, blood, etc. (Gould, 1958, p. 256).

An enzyme, cholesterol acyltransferase in the liver or plasma, works in esterification of cholesterol. One of the pathways is the esterification by transferring fatty acids from lecithin. From this way of esterification, the lecithin becomes lysolecithin, which has been reported to be increased in the blood of women on contraceptive hormone therapy (Wld. Hlth. Org. Rep. Ser. 527, 1971, p. 10).

From the above, it seems that the contraceptive steroids causes an increased lectithin synthesis, and also the esterification of cholesterol with lecithin. So, an elevated body tissue cholesterol esters might be expected by treatment with female sex hormones.
EXPERIMENTAL PROCEDURE

Experimental design

Forty-nine female rats averaging 95 grams body weight were divided into four groups (see Table 1). The four groups were treated as follows:

Twelve rats were fed 2 ppm of dietary zinc with placebo injection, another 12 rats were fed 140 ppm of dietary zinc with placebo injection, the third group of 13 rats were fed 2 ppm of dietary zinc with contraceptive steroid injection and the last group of 12 rats were fed 140 ppm of dietary zinc with contraceptive steroid injection.

Table 1. Experimental design

<table>
<thead>
<tr>
<th>Dietary Group</th>
<th>Hormone treatment</th>
<th>Number of animals</th>
<th>Zinc level</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zn deficient</td>
<td>placebo injected</td>
<td>12</td>
<td>2 ppm</td>
</tr>
<tr>
<td>Zn deficient</td>
<td>contraceptive steroids injected</td>
<td>12</td>
<td>2 ppm</td>
</tr>
<tr>
<td>Zn supplemented</td>
<td>placebo injected</td>
<td>13</td>
<td>140 ppm*</td>
</tr>
<tr>
<td>Zn supplemented</td>
<td>contraceptive steroids injected</td>
<td>12</td>
<td>140 ppm*</td>
</tr>
</tbody>
</table>

* analyzed value of zinc level in the diet of zinc supplemented groups

Contraceptive steroid injections consisted of 0.1 ug of Ethynyl Estradiol and 0.1 mg of Medroxyprogesterone (17-Hydroxy-6a-methyl pregn-4-ene-3,20-dione) dispersed in 0.1 ml of corn oil. The non-hormone
treated rats received 0.1 ml of plain corn oil. Injections were administered intramuscularly.

**Animal care**

Rats were housed individually in stainless steel cages with wire screen bottoms. The cages, feed cups and water bottles were rinsed with EDTA (ethylenediaminetetraacetic acid). Rats fed the zinc deficient diets were caged on the top rows and were curtained with vinyl to minimize zinc contamination from the environment. Distilled water was allowed as needed.

**Diet preparation**

The composition of the diets are presented in Table 2. During the preparation of the diet, zinc contamination was minimized by rinsing the utensils with EDTA and by wearing disposable plastic gloves.

The different mineral elements were weighed and made into homogeneous mixture in a stainless steel mixing bowl. The ingredients were measured and mixed in a large stainless steel bowl on an electric mixer; then corn oil was added and mixed thoroughly. The diet was stored in a refrigerator. For the zinc supplementation ZnSO$_4$$ \cdot 7H_2O$ was used. The zinc level of the zinc deficient diets by analysis was 2 ppm and 140 ppm of zinc for the supplemented diets.

**Collection of samples**

Food intake and weight gain were determined weekly. Blood samples were drawn weekly, serum was separated and serum zinc levels determined.
Table 2. Basal diet composition with mineral mix

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>g/100g of diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>59.00</td>
</tr>
<tr>
<td>Dried Egg White</td>
<td>20.00 (For only Zn supplemented diet)</td>
</tr>
<tr>
<td>Corn Oil</td>
<td>10.00</td>
</tr>
<tr>
<td>EDTA-Washed Casein</td>
<td>20.00 (For only Zn deficient diet)</td>
</tr>
<tr>
<td>-Cellulose</td>
<td>5.00</td>
</tr>
<tr>
<td>Mineral Mix*</td>
<td>4.00</td>
</tr>
<tr>
<td>Vitamin Mix*</td>
<td>2.00</td>
</tr>
</tbody>
</table>

Mineral Mixture

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>g/kg of Mineral Mixture</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na₂HPO₄ *H₂O</td>
<td>323</td>
</tr>
<tr>
<td>CaCO₃</td>
<td>312</td>
</tr>
<tr>
<td>KCl</td>
<td>86</td>
</tr>
<tr>
<td>MgCO₃</td>
<td>34.7</td>
</tr>
<tr>
<td>FeSO₄ *7H₂O</td>
<td>4.3</td>
</tr>
<tr>
<td>MnSO₄</td>
<td>3.8</td>
</tr>
<tr>
<td>CoCl</td>
<td>0.5</td>
</tr>
<tr>
<td>CuSO₄ *H₂O</td>
<td>0.4</td>
</tr>
<tr>
<td>Na₂MoO₄</td>
<td>0.06</td>
</tr>
<tr>
<td>KI</td>
<td>0.05</td>
</tr>
</tbody>
</table>

* Vitamin Mix used is Vitamin diet Fortification Mixture, Nutritional Biochemical Corporation, Cleveland, Ohio (Recka, Dalvi, 1971, p. 55).
200 microletters of serum were diluted to 1 ml with 0.2 N nitric acid. Zinc concentration was determined by atomic absorption Spectrometry.

After eight weeks of the experimental treatment, the rats were fasted for 12 hours before they were killed. The final body weights were recorded and they were killed by decapitation.

Livers, adrenals and ovaries were removed, weighed and immediately frozen in absolute ethanol with dry ice in it. The frozen tissues were stored at -20°C until further analyses were made. The remaining carcasses minus the intestinal tract were placed in individually numbered jars, autoclaved and were then homogenized. When each carcass was homogenized, the blender and other equipment was rinsed with EDTA solution.

Methods of assays

Determination of zinc levels. The crucibles for ashing samples were previously rinsed with HCl (hydrochloric acid). After filling the crucibles with HCl, they were allowed to boil for 3 to 5 minutes on the hot plate. After discarding the acid, they were rinsed with deionized water. The clean crucibles were dried and stored in a descicator. The weights of the crucibles were recorded.

Zinc in the diet. Approximately two grams of diet were weighed into the previously washed crucibles, and were charred on a hot plate for a few hours. Then they were ashed overnight in a muffle furnace at 600°C. Five ml of concentrated nitric acid was added into each crucible to dissolve the ash. The dissolved ash was transferred into screw capped bottles which were previously acid rinsed, and filled with triple distilled water to 100 grams. Zinc level was measured by atomic absorption spectrophotometry.
Serum zinc level. Blood was taken from the retro ocular sinus with a capillary tube, and was allowed to clot. The serum was separated. Two tenths ml of serum was diluted to 1 ml with 0.2-N-Nitric acid. The zinc concentration was determined by atomic absorption spectrophotometer. The solutions were aspirated into an air: acetylene flame and the atomic absorption of zinc was measured at the 213.9 nm wave length.

Carcass ash, zinc and calcium levels. Approximately two grams of autoclaved and homogenized carcass was weighed into clean acid rinsed crucibles and was ashed for 24 hours in a muffle furnace at 600°C. The net ash contents were calculated. The percentage of carcass ash was determined as indicated below:

Percentage of ash in wet matter = \( \frac{\text{net weight of ashed sample}}{\text{net weight of wet sample}} \times 100 \)

Percentage of ash in dry matter = \( \frac{\text{net weight of ashed sample}}{\text{weight of wet sample} \times \% \text{ of dry matter}} \times 100 \)

The ash was dissolved with 5 ml of distilled (concentrated) nitric acid, which was transfered into acid rinsed bottles with screw caps. Final weight was brought to 100 grams with deionized water. Carcass zinc level was determined on ashed samples by atomic absorption spectrophotometry.

Determination of body composition. For water content a 20 gram sample of the homogenized carcass was weighed into pre-weighed and numbered beakers. Carcass samples were dried in an oven at 105°C overnight until it came to a constant weight. Beakers with the dried sample were cooled and weighed. The percentage of water content was calculated as below:
Percentage of water = \frac{\text{weight of wet sample} - \text{weight of dried sample}}{\text{weight of wet sample}} \times 100

**Total lipids.** The dry material from the determination of the water content was used for lipid analysis. About 75 ml of 1:1 acetone, ethyl-ether mixture was added to the beaker and the contents stirred. The beaker was capped with a tight-fitting aluminum cover and allowed to stand overnight. All supernatant extracts were collected from each sample and allowed to evaporate. This procedure was repeated twice for a total of three extractions. If the third extract was still dark, a fourth extraction was made. After the final extraction, the final traces of ether are allowed to evaporate and the material is then dried overnight at 105°C. After cooling in a desiccator the samples were weighed.

Percentage of lipid in carcass = \frac{\text{Wt. of dry tissues} - \text{Wt. of fat-free tissues}}{\text{Wt. of wet tissues}} \times 100

The Iodine number of carcass lipids extracts was determined by the Hanus Method (see Appendix A).

**Determination of carcass cholesterol ester level**

The carcass lipids extracted from the homogenized carcasses were fractionated by the methods of Hirsh and Ahrena (1958) as outlined in Appendix B.

A simplified colorimetric method was applied for chemical examination of serum cholesterol levels. Liebermann-Burchard reagent was prepared as follows: cool acetic anhydride and concentrated sulfuric acid in ice water or in the freezer compartment. To a 500 ml amber
glass bottle, fitted with a polyseal cap, add 220 ml of cold acetic anhydride and 200 ml of glacial acetic acid. Mix by inversion and add 30 ml of cold concentrated sulfuric acid. The reagent was stored at 4°C in the dark.

HYCEL Cholesterol Standard, which is 0.2% cholesterol in glacial acetic acid, which is equivalent to 200 mg% cholesterol in serum, was used.

Similar amounts of lipid extract from each of the four groups were weighed into test tubes. One ml of isopropyl alcohol was added into each tube which was then capped and shaken vigorously for 30 seconds on the vortex mixer. Tubes were allowed to stand 5 minutes, again were shaken for a full 30 seconds. Then, they were centrifuged at 1500 R.P.M. for 10 minutes. Supernatant extracts were placed into 50 ml beakers. One percent methanolic digitonin in the amount of 0.5 ml, and 0.5 ml of distilled water were added, and mixed by gently swirling. Beakers were placed on a steam bath (or moderate hot plate) and evaporated to dryness and baked about 1 to 2 minutes until absolutely dry. Two ml acetone was added and the residue was extracted by gentle swirling. The acetone layer was transferred into test tubes. The extraction procedure with acetone was repeated twice more. The tubes were placed in boiling water bath and evaporated to dryness. One hundred lambda glacial acetic acid was added to each tube. Liberman-Burchard reagent was added in the amount of 6 ml into each tube, and mixed well on the vortex mixer. The Standard tube contained 0.1 ml HYCEL cholesterol Standard and 0.1 ml of glacial acetic acid; the blank tube had 0.1 ml glacial acetic acid. Test tubes were placed in a water bath at 37°C for 20 minutes, after which optical density was measured at 625mu wave
length within 10 minutes. The cholesterol level was calculated as follows:

\[
\text{mg/100 ml of sample} = \frac{\text{O.D. of sample}}{\text{O.D. of standard}} \times \frac{\text{mg/100 ml of standard}}{\text{mg/100 ml of sample}}
\]

HYCEL Cholesterol Standard was purchased from HYCEL, Inc.

**Determination of total cholesterol levels in adrenals, ovaries and livers**

The frozen tissues were removed from the freezer and allowed to thaw. Approximately 10 mg of ovaries, 15 mg of adrenals and 200 mg of liver tissues were weighed and homogenized in a glass homogenizer with small volume of solvent. Solvent mixture (acetone:ethanol is 1:1) was added to be about 25 parts, rinsing pestle with solvents. The homogenizer was capped and was shaken vigorously for 30 seconds and the homogenate was filtered through glass wool into test tubes. The test tubes were cooled, 1 ml of solvent was added to each tube to make the final volume 2 ml of lipid extract. The extracts were placed in a hot water bath and evaporated to dryness. After cooling them, 0.1 ml of glacial acetic acid and Lieberman-Burchard reagent were added. The method for cholesterol was followed from this point.

**Statistical analysis**

Conventional statistical methods were used for evaluation of the data. Figures in the tables except for serum zinc level, expressed in group means plus or minus standard deviations. Student T test was applied to compare the means of the four groups of zinc deficient, zinc deficient-hormone injected, zinc supplemented, and zinc supplemented-hormone injected rats. In addition to the student t test, Least Significant Difference was used.
RESULTS

Dietary zinc deprivation of rats resulted in a failure in growth. This was probably precipitated by anorexia observed in the zinc deficient rats. Zinc deficient rats kept their paws close together arching their backs, and their hair was rough and looked unkept. The toes of zinc deficient rats were swollen and red. Skin lesions appeared on both sides of the mouth with red thickened epithelium.

After a week of the experimental treatment, three rats from the zinc deficient and hormone injected group had severe signs of skin lesions. And about a week later, zinc deficient rats with placebo injections, started to show these same kinds of lesions. At the end of the third week of the experiment, two rats from the zinc deficient hormone injected group died. After the fourth week, skin lesions remained at the same severity, but the zinc deficient non-hormone treated rats were getting worse little by little.

Vaginal smears were examined, and ovulation was inhibited in all rats injected with contraceptive steroids. The rats fed the zinc deficient diet did not cycle, either.

Growth rate

Table 3 and Figure 1 represent the growth patterns of the four groups at weekly intervals.

The zinc deficient rats ate markedly less. From the first week of the experimental diet, their intake was about one half that of zinc
Table 3. Effect of dietary zinc and contraceptive steroid treatment on growth of female rats

<table>
<thead>
<tr>
<th>Weeks</th>
<th>Initial (g)</th>
<th>1 (g)</th>
<th>2 (g)</th>
<th>3 (g)</th>
<th>4 (g)</th>
<th>5 (g)</th>
<th>6 (g)</th>
<th>7 (g)</th>
<th>8 (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zn deficient</td>
<td>85±6*</td>
<td>99±9*</td>
<td>105±9a</td>
<td>106±9a</td>
<td>102±27a</td>
<td>107±9a</td>
<td>111±10a</td>
<td>111±10a</td>
<td>107±9a</td>
</tr>
<tr>
<td>Zn deficient &amp; Hormone Inj.</td>
<td>98±7</td>
<td>105±7</td>
<td>101±3</td>
<td>109±11</td>
<td>108±9</td>
<td>106±10</td>
<td>105±10</td>
<td>106±11</td>
<td>99±8</td>
</tr>
<tr>
<td>Zn supplemented</td>
<td>97±7</td>
<td>129±8</td>
<td>151±7</td>
<td>173±10</td>
<td>192±28</td>
<td>205±15</td>
<td>215±15</td>
<td>225±13</td>
<td>221±19</td>
</tr>
<tr>
<td>Zn supplemented &amp; Hormone Inj.</td>
<td>94±6</td>
<td>128±14b</td>
<td>143±14b</td>
<td>156±48b</td>
<td>180±19b</td>
<td>195±22b</td>
<td>201±25b</td>
<td>209±22b</td>
<td>199±27b</td>
</tr>
</tbody>
</table>

* denote mean ± standard deviation

a indicate significant difference between zinc supplemented and zinc deficient groups ($\alpha = 0.01$)

b indicate significant difference between zinc supplemented group and zinc deficient group with steroid injections ($\alpha = 0.01$)
Figure 1. Effect of dietary zinc level and contraceptive hormone treatment on growth of female rats
mean ± S.D.
supplemented rats. Throughout the experiment this lower diet consumption remained constant. At the conclusion of the experiment, the mean body weight gained of the zinc deficient rats was 22.2 grams, and that of the rats zinc deficient with hormone treatment was about one gram.

The food efficiency for weight gain is shown in Table 4. Rats fed the zinc deficient diet showed a reduced food efficiency. The overall value of food efficiency was 10.2 for zinc deficient rats, and 4.1 for zinc supplemented rats. Hormone treatment slightly improved the appetite; the zinc deficient rats ate around 46.3 grams per week, while the zinc deficient and hormone injected rats ate 52.2 grams weekly. This did not, however, result in an accompanying body weight increase. Consequently the food efficiency of the hormone treated rats was lower than that of the non-hormone injected rats.

**Serum zinc level**

The weekly fluctuation of serum zinc levels are presented in Figure 2 and Table 5.

The range of the initial serum zinc level was from 155 ug% to 170 ug%. After two weeks of treatment serum zinc in the zinc supplemented rats rose to 200 ug%, while the zinc deficient group was reduced to 50 ug%. The lowest value of 35 ug% appeared in the zinc deficient, contraceptive steroid injected group, and which was followed by a slight increase up to 60 ug% at the fifth week of the experimental treatment.

In both zinc supplemented and deficient groups, contraceptive steroid injection caused a slightly lowered serum zinc level compared
Table 4. Effect of dietary zinc and contraceptive steroid treatment on food consumption, weight gain and food efficiency of female rats

<table>
<thead>
<tr>
<th>Weeks</th>
<th>2nd</th>
<th>3rd</th>
<th>4th</th>
<th>5th</th>
<th>6th</th>
<th>7th</th>
<th>8th</th>
<th>Diet consumed</th>
<th>B.W. gained**</th>
<th>Food efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zn deficient</td>
<td>46.0</td>
<td>42.4</td>
<td>46.3</td>
<td>49.2</td>
<td>51.0</td>
<td>48.4</td>
<td>45.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>6.3</td>
<td>0.3</td>
<td>-</td>
<td>0.7</td>
<td>0.8</td>
<td>-0.8*</td>
<td>-6.0*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>7.3</td>
<td>128.5</td>
<td>-</td>
<td>70.3</td>
<td>63.7</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zn deficient</td>
<td>50.2</td>
<td>43.6</td>
<td>45.5</td>
<td>46.1</td>
<td>49.7</td>
<td>54.3</td>
<td>54.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&amp; Hormone Inj.</td>
<td>4.2</td>
<td>0.2</td>
<td>-1.0*</td>
<td>-0.2*</td>
<td>-0.3*</td>
<td>-0.9*</td>
<td>-7.4*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>11.9</td>
<td>189.4</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zn supplemented</td>
<td>89.5</td>
<td>85.6</td>
<td>104.9</td>
<td>-</td>
<td>106.7</td>
<td>106.4</td>
<td>92.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>23.1</td>
<td>22.3</td>
<td>16.3</td>
<td>-</td>
<td>9.8</td>
<td>8.2</td>
<td>-0.9*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>3.8</td>
<td>3.8</td>
<td>6.4</td>
<td>-</td>
<td>10.9</td>
<td>13.0</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zn supplemented</td>
<td>96.0</td>
<td>85.2</td>
<td>105.6</td>
<td>-</td>
<td>105.6</td>
<td>105.7</td>
<td>89.1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&amp; Hormone Inj.</td>
<td>23.3</td>
<td>19.7</td>
<td>11.4</td>
<td>-</td>
<td>7.8</td>
<td>7.8</td>
<td>-5.2*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>4.1</td>
<td>4.3</td>
<td>9.2</td>
<td>-</td>
<td>13.3</td>
<td>13.6</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Negative sign indicates loss of body weight
** Body weight per rat per week
Table 5. Effect of dietary zinc level and contraceptive steroid treatment on serum zinc levels of female rats

<table>
<thead>
<tr>
<th>Weeks</th>
<th>Number of Samples</th>
<th>Initial</th>
<th>2nd Week</th>
<th>3rd Week</th>
<th>5th Week</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zn deficient</td>
<td>(6)</td>
<td>165&lt;sup&gt;1&lt;/sup&gt;,*</td>
<td>65</td>
<td>45</td>
<td>63</td>
</tr>
<tr>
<td>Zn deficient &amp; Hormone Inj.</td>
<td>(6)</td>
<td>155</td>
<td>50</td>
<td>35</td>
<td>60</td>
</tr>
<tr>
<td>Zn supplemented</td>
<td>(6)</td>
<td>170</td>
<td>205</td>
<td>250</td>
<td>213</td>
</tr>
<tr>
<td>Zn supplemented &amp; Hormone Inj.</td>
<td>(6)</td>
<td>160</td>
<td>223</td>
<td>194</td>
<td>205</td>
</tr>
</tbody>
</table>

Least Significant Difference (0.025)

34 19 10

<sup>1</sup> Mean expressed as microgram per 100 ml of serum

* Means which are pooled
Figure 2. Effect of dietary zinc level and contraceptive hormone treatment on serum zinc levels of female rats.
to the levels of the non-hormone treated groups. These differences were not statistically significant but were rather consistent.

**Organ weight and total cholesterol contents**

Table 6 shows the weight of adrenals and ovaries and total cholesterol contents.

Zinc deficiency did not cause notable changes in relative adrenal weight. However, deficiency caused an elevation of the cholesterol content of the adrenals. Contraceptive steroid injection reduced the total adrenal weight. The total adrenal weight of hormone injected rats of both zinc supplemented and deficient groups, were almost the same, however, when expressed as a percentage of the body weight, animals fed the zinc deficient diet had significantly larger adrenals.

Zinc deficiency resulted in a decreased ovarian size, which was further decreased by hormone injection. The total cholesterol content of ovaries from zinc deficient rats was higher than that of the zinc supplemented rats. Contraceptive steroid injections induced an increase in total cholesterol content of the ovaries.

The liver weights per 100 grams of body weight did not show significant differences among the four groups, although liver weights tended to be increased by contraceptive steroid injections.

Liver lipids in terms of percentage on a dry matter basis (Table 7) was significantly decreased by feeding the zinc deficient diet. There was a tendency for liver lipids to be increased by contraceptive steroids.

Total liver cholesterol contents were not statistically different among the four groups (especially, when homogenized fresh tissue was
Table 6. Effect of dietary zinc level and contraceptive steroid treatment on the weights and total cholesterol contents of adrenals and ovaries from female rats

<table>
<thead>
<tr>
<th></th>
<th>Number of Samples</th>
<th>Total Weight (mg)</th>
<th>mg/100g of B.W.</th>
<th>Total Cholesterol level (mg%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Adrenals</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zn deficient</td>
<td>(11)</td>
<td>22.4±3.3*</td>
<td>20.3±3.2</td>
<td>20.2±5.2</td>
</tr>
<tr>
<td>Zn deficient &amp; Hormone Inj.</td>
<td>(11)</td>
<td>10.9±2.3</td>
<td>13.0±2.3</td>
<td>-</td>
</tr>
<tr>
<td>Zn supplemented</td>
<td>(11)</td>
<td>43.8±5.5</td>
<td>19.5±2.5</td>
<td>11.2±2.5</td>
</tr>
<tr>
<td>Zn supplemented &amp; Hormone Inj.</td>
<td>(11)</td>
<td>11.3±1.5</td>
<td>6.5±0.7</td>
<td>18.2±3.8</td>
</tr>
<tr>
<td>LSD (0.05/0.01)</td>
<td></td>
<td>10.2/15.2</td>
<td>3.2/5.4</td>
<td>3.6/5.6</td>
</tr>
<tr>
<td><strong>Ovaries</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zn deficient</td>
<td>(10)</td>
<td>15.7±2.2</td>
<td>14.2±2.1</td>
<td>6.4±1.2</td>
</tr>
<tr>
<td>Zn deficient &amp; Hormone Inj.</td>
<td>(10)</td>
<td>9.1±2.1</td>
<td>8.6±2.1</td>
<td>7.9±2.6</td>
</tr>
<tr>
<td>Zn supplemented</td>
<td>(10)</td>
<td>44.6±2.5</td>
<td>20.0±1.1</td>
<td>3.1±0.2</td>
</tr>
<tr>
<td>Zn supplemented &amp; Hormone Inj.</td>
<td>(10)</td>
<td>16.5±2.7</td>
<td>8.0±1.4</td>
<td>6.0±0.5</td>
</tr>
<tr>
<td>Least Significant Difference (0.05/0.01)</td>
<td></td>
<td>5.9/8.8</td>
<td>2.3/3.4</td>
<td>1.0/1.5</td>
</tr>
</tbody>
</table>

* Mean ± S.D.
Table 7. Effect of dietary level and contraceptive steroid treatment on liver lipids of female rats

<table>
<thead>
<tr>
<th>Number of Samples</th>
<th>% of Body Wt.</th>
<th>Total Lipid % on Dry Matter Basis</th>
<th>Total Cholesterol mg %</th>
<th>Free Cholesterol mg %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zn deficient</td>
<td></td>
<td>2.7±0.1\textsuperscript{a}(0.05)</td>
<td>5.0±0.9\textsuperscript{a}(0.05)</td>
<td>1.1±0.1</td>
</tr>
<tr>
<td>Zn deficient &amp; Hormone Inj.</td>
<td>(8)</td>
<td>3.2±0.6\textsuperscript{b}(0.025)</td>
<td>6.8±1.9\textsuperscript{b}(0.005)</td>
<td>1.2±0.2\textsuperscript{**b}(0.005)</td>
</tr>
<tr>
<td>Zn supplemented</td>
<td></td>
<td>2.5±0.2\textsuperscript{c}(0.05)</td>
<td>12.0±1.5\textsuperscript{c}(0.005)</td>
<td>1.3±0.1\textsuperscript{c}(0.05)</td>
</tr>
<tr>
<td>Zn supplemented &amp; Hormone Inj.</td>
<td>(9)</td>
<td>2.8±0.2\textsuperscript{d}(0.005)</td>
<td>14.9±2.8\textsuperscript{d}(0.1)</td>
<td>1.6±0.2\textsuperscript{d}(0.05)</td>
</tr>
</tbody>
</table>

* Mean ± S.D.

** Figures + parenthesis denote \( t \) for the critical value of \( t \)

a significant difference in comparison with zinc deficient and zinc deficient with hormone injection

b significant difference in comparison with zinc deficient with hormone injection and zinc supplemented with hormone injection

c significant difference in comparison with zinc deficient and zinc supplemented

d significant difference in comparison with zinc supplemented and zinc supplemented with hormone injection
used for the determination of the total cholesterol level). Contraceptive steroid injection resulted in elevated cholesterol levels in both zinc supplemented and zinc deficient rats.

**Body composition**

Carcass water, total lipids, cholesterol esters, and ash contents are summarized in Tables 8, 9 and 10.

Animals fed the zinc deficient diet had a greater percentage of carcass water than those fed the zinc supplemented diet. Contraceptive steroids injection caused a slight but insignificant decrease in the percentage of carcass moisture content.

Total carcass lipids were significantly decreased in animals fed the zinc deficient diet. The steroid hormone injection to the zinc deficient rats resulted in an elevated body lipid compared to the non-injected zinc deficient rats. This hormone injection caused a decrease in the percentage of lipids in zinc supplemented rats.

Zinc deficient rats had markedly elevated carcass cholesterol esters. Contraceptive steroids injection caused a slight increase in carcass cholesterol esters (Table 10).

Table 9 presents the percentage of carcass ash on fat free basis with zinc and calcium contents per mg of carcass ash. Carcass ash was increased in zinc deficient rats on the dry, fat free basis. Hormone injection elevated carcass ash content with an increase in its zinc and calcium content.
Table 8. Effect of dietary zinc level and contraceptive steroid treatment on carcass composition of female rats

<table>
<thead>
<tr>
<th>Number of Samples</th>
<th>Moisture %</th>
<th>Ash (%)</th>
<th>Lipid %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Dry Matter Basis</td>
<td>Wet Matter Basis</td>
</tr>
<tr>
<td>Zn deficient</td>
<td>(11)</td>
<td>67.2±0.7*a(0.005)</td>
<td>1.7±0.2</td>
</tr>
<tr>
<td>Zn deficient &amp; Hormone Inj.</td>
<td>(12)</td>
<td>58.0±1.3b(0.01)</td>
<td>2.4±0.1</td>
</tr>
<tr>
<td>Zn supplemented</td>
<td>(13)</td>
<td>61.2±2.1c(0.005)</td>
<td>1.5±0.0</td>
</tr>
<tr>
<td>Zn supplemented &amp; Hormone Inj.</td>
<td>(10)</td>
<td>60.2±1.3</td>
<td>1.8±0.2</td>
</tr>
</tbody>
</table>

* Mean ± S.D.

** Figures within the parenthesis are for the critical values of t.

a significant difference in comparison with zinc deficient and zinc deficient with hormone injection

b significant difference in comparison with zinc deficient with hormone injection and zinc supplemented with hormone injection

c significant difference in comparison with zinc deficient and zinc supplemented

d significant difference in comparison with zinc supplemented and zinc supplemented with hormone injection
Table 9. Effect of dietary zinc level and contraceptive steroids treatment of carcass minerals of female rats

<table>
<thead>
<tr>
<th>Ash (%)</th>
<th>Zn (ug/g ash)</th>
<th>Ca (ug/g ash)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dry &amp; Fat Free Basis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zn deficient</td>
<td>6.2±0.6*</td>
<td>1.6±0.4</td>
</tr>
<tr>
<td>Zn deficient &amp; Hormone Inj.</td>
<td>7.0±0.6</td>
<td>2.1±0.2</td>
</tr>
<tr>
<td>Zn supplemented</td>
<td>5.1±0.4</td>
<td>3.2±0.2</td>
</tr>
<tr>
<td>Zn supplemented &amp; Hormone Inj.</td>
<td>6.0±0.7</td>
<td>4.8±0.1</td>
</tr>
<tr>
<td>Least Significant Difference (0.05/0.01)</td>
<td>1.6/2.4</td>
<td>0.5/0.7</td>
</tr>
</tbody>
</table>

* Figures denote mean ± S.D.
Table 10. Characterization of carcass lipids from female rats fed different levels of zinc and with or without contraceptive steroid treatment

<table>
<thead>
<tr>
<th>Number of Samples</th>
<th>Total Lipid % Dry Matter Basis</th>
<th>Cholesterol Esters % of Lipid</th>
<th>Iodine Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zn deficient (6)</td>
<td>14.4±0.8*</td>
<td>2.67**</td>
<td>66**</td>
</tr>
<tr>
<td>Zn deficient (6)</td>
<td>19.5±1.3</td>
<td>3.67</td>
<td>59</td>
</tr>
<tr>
<td>&amp; Hormone Inj.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zn supplemented (6)</td>
<td>25.4±4.2</td>
<td>1.10</td>
<td>60</td>
</tr>
<tr>
<td>Zn supplemented (6)</td>
<td>23.8±4.4</td>
<td>1.50</td>
<td>57</td>
</tr>
<tr>
<td>&amp; Hormone Inj.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Least significant Difference (0.05/0.01)</td>
<td>4.9/7.3</td>
<td>0.43/0.64</td>
<td></td>
</tr>
</tbody>
</table>

* Figures denote mean ± S.D.

** Figures are pooled
DISCUSSION AND CONCLUSIONS

Food intake

The markedly decreased food consumption appears very quickly (within a week in this experiment) in animals fed the zinc deficient diet. Anorexia, which is the very first and dominating symptom of the zinc deficiency, has been observed in several species when a zinc deficient diet is fed. Fox and Harrison (1966) reported altered plasma protein levels in zinc deficient rats, i.e., that plasma albumin was significantly reduced. These researchers felt this may explain the decreased appetite in the zinc deficient rats. Quarterman and Chesters, (1973) mentioned that zinc deficient diet causes an impairment of a metabolism resulting in an accumulation of certain metabolites in body tissues which may cause anorexia in the zinc deficient animals. Small for age children have been studied with very similar findings of low hair zinc level, an impaired taste acuity, and less appetite in comparison with normal weight children of the same age (Hambidge et. al., 1972). However, those children's diet was not analyzed.

Insufficient food intake seems to be the most responsible for the inhibited growth (weight gain) in zinc deficient animals. The size of zinc deficient rats was half of the zinc supplemented rats.

Weight gain and body composition

Consumption of a zinc deficient diet is known to result in retarded growth of animals. Lucille S. Hurley (1969) and Richard H. Follis, Jr., (1966), for instance, reported a reduced weight gain in rats fed zinc
deficient diets. Weanling rats were fed zinc deficient diet for 12 to 14 weeks. The rats gained about 60 grams, and they stopped growing at this body weight. In this experiment the initial body weight was between 80 to 95 grams: zinc deficient diet treatment kept their body weight almost constant, while zinc supplementation of the diet resulted in as much as 100 grams of body weight gain (their final weight was around 200 grams, Table 3).

When compared to the effect of zinc deficient diet in gain in body weight, contraceptive steroid treatment showed less influence on body weight (Table 3). As McBean (1971) and Young (1971) reported, estrogen treated rats weighed less than controls. Frye (1967) mentioned an inhibitory effect of estrogen on growth of young animals. However, a more thorough study such as that of David Young's (1971), for example, has to be done in order to explain the relation between reduced weight gain and estrogen therapy. From his study it was reported that estrogen treatment decreased the activity of diglyceride acyltransferase, which is the enzyme for triglyceride synthesis by the liver. This might be responsible for the slower gain in body weight than controls in his study; however, which may only mean less body lipid deposition.

In this study, it was attempted to determine the composition of the gained or lost body weight by analyzing total body composition.

**Serum zinc and certain steroid producing organs**

When dietary zinc cannot meet the body requirement, the zinc in the body pools including serum zinc will be used to meet the deficit. So the diet deficient in zinc, will result in a decreased serum zinc
level. Supposedly the stress of zinc deficiency which causes various clinical disorders, might work together to lower the serum zinc, where the mechanism is not known.

Besides the dietary effect on the regulation of serum zinc level, hormones and serum protein concentration have been mentioned to influence serum zinc. Lowered serum zinc level has been found in the subjects with several diseases and in women who are pregnant and who are on oral contraceptives (Halsted and Smith, Jr., 1970).

A hormonal regulation on serum zinc level is a recent topic. Oral contraceptive treatment, generally, increases the level of circulating corticosteroids, which reduces serum zinc (McBean, 1971; Halsted and Smith, Jr., 1970). Also, corticosteroid treatment reduces serum zinc (Flynn et. al., 1972). Table 5 shows a tendency toward a decreased serum zinc by hormone injection, which was not statistically significant. Flynn and his associates (1972) stated that corticotropin activity of the adrenal cortex is an indicator of alteration in serum zinc.

How does the reduced serum zinc in zinc deficient groups influence the adrenals or other steroid producing tissues? How are those organs functioning? Except that the adrenals contain appreciable zinc, it is not clearly understood how zinc is related to adrenals and vice versa.

In Table 6, the adrenal weights of zinc deficient and zinc supplemented rats are not significantly different, although, zinc deficient rats had slightly larger mean adrenal weight. However, the cholesterol content of both adrenals and ovaries was very different as a consequence of dietary treatment. Cholesterol level of these organs was almost twice as much in the zinc deficient animals as the zinc supplemented ones.
Experimental zinc deficient animals always show clinical manifestations of zinc deficiency. This stress could stimulate adrenals to release adrenocorticotrophin. Macapinlac et. al. (1966) suggested that this condition may produce a hyperactive adrenal.

Quarterman and his colleagues (1972) reported a slightly increased adrenal weight in zinc deficient rats, which is accompanied with an increased cholesterol and ascorbic acid content. This finding indicates that these organs do not function normally (hypofunction).

When adrenals do not respond efficiently to produce adrenal steroid hormones and/or when the degradation of steroids hormone are accelerated, then, the resulted low circulating steroid level will cause a continuous stimulation on the pituitary gland to release ACTH. Particularly, when adrenals are not capable of meeting the stimulation to release (or synthesis) adrenal steroid hormones into circulation, a nonfunctional enlargement of adrenals might be produced by the condition stated above. For instance, removal of ACTH stimulation by hypophysectomy, results in a decreased activity of cholesterol-esterase in adrenals, which is responsible for an increased cholesterol content in the adrenals hypophysectomized rats.

Similarly, administration of contraceptive hormones has been shown to increase and maintain a high level of circulating corticosteroids and circulating female sex hormones. These elevated steroid hormones will shut off the further stimulation of adrenals and ovaries by pituitary hormones, through the negative feedback regulatory mechanism. Consequently, this treatment results in an inactive state of those tissues; smaller size of ovaries with increased cholesterol contents has been shown (Moghissi, 1973).
The pituitary gonadotrophins, Follicular Stimulating Hormone and Luteinizing Hormone and/or Prolactin, stimulate ovaries to produce female sex hormones as it also stimulates the growth and maturation of ovaries. Gonadotrophin treatment caused a depletion of cholesterol ester to produce ovarian steroids in ovaries of rats (Behrman and Greep, 1972).

At this point, it may be summarized that zinc deficient diet through some unknown mechanism, causes an interference on certain enzyme system for the consumption of cholesterol being converted to steroids. And this could be as similar condition as the continuous steroid injection shuts off the stimulation from pituitary to keep the steroid producing organs nonfunctional.

One of the well-known symptoms of zinc deficiency is undergrowth in general, specially hypogonadism. For instance, a decreased pituitary gonadotrophins in the zinc deficient dwarfed boys (Prasad et al., 1973) and underdeveloped gonads in zinc deficient animals (Sandstead et al., 1966) were reported. Therefore, the decreased ovarian weights and increased cholesterol content in the zinc deficient rats (Table 6) may indicate an insufficient release of FSH and LH. Zinc deficient rats did not ovulate during the experiment.

Carcass composition

Some biochemical studies have been done on the dependency of certain enzymes on zinc. A reduced proliferative activity of cartilage cells was reported in the rats fed zinc deficient diet (Follis et al., 1941). This activity is necessary for the metabolism of collagen with proteins of hydroxy proline and its peptides. Griffith and Alexander (1972) reported that the rats fed a zinc deficient diet showed an
abnormally high urinary excretion of these hydroxylated amino acids. According to these two studies, it may be summarized that zinc deficient diet causes a defect on collagen metabolism. Is this then a reason for inhibited growth in rats fed a zinc deficient diet?

Since pituitary trophic hormones have trophic effects on certain body organs a failure to produce these hormones may result in undergrowth of its target organs and animal growth on the whole.

During the first two weeks of this experiment (Follis et. al., 1966) zinc deficient rats consumed about 88 grams of diet and gained about 6.6 grams in body weight while the rats with steroid injection ate about 93 grams with about 4.2 grams of body weight gained (figures are pooled data). Zinc supplemented rats, with hormone injection, ate about 180 grams of diet and gained 45.2 grams; the rats without hormone injection consumed 175 grams and raised their body weight as much as 42.8 grams. Contraceptive, steroid injections resulted in lowered food efficiency (Table 4).

Retarded body weight gain has been shown to be caused by contraceptive steroids therapy (McBean, 1971). Since triglycerides are the major component of the storage fat, reduced synthesis of this lipid by oral contraceptive hormone treatment (Young, 1972) may decrease body fat mass with an increase in lean body mass (Garcia, Goldzieher and Massey, 1973). The hormone insulin has a powerful anabolic effect on fat synthesis. An elevated circulating insulin level is a general finding in oral contraceptive users. However, its activity has been a subject of discussion. Spellacy (1973) suggested that the increased insulin might be an inactive form of proinsulin and might be antagonized by certain metabolites such as xanthuronic acid which is elevated by estrogen treatment.
The administration of contraceptive steroids was reported to produce pyridoxine deficiency (Nutrition Review, 1973). Pyridoxine serves as a cofactor in the pathway of conversion of the amino acids tryptophan into nicotinic acid. Therefore, as a result, this steroid therapy increases the formation of the intermediate metabolite, xanthuronic acid. Xanthuronic acid is said to bind insulin molecules and inhibits its biological action (Spellacy, 1973). Contraceptive injection caused a decrease in percentage of carcass lipids in zinc supplemented rats (Table 10), although it was not statistically significant. The same treatment, however, showed a tendency to increase body lipids in zinc deficient rats.

A deleterious effect of a zinc deficient diet on the physiology of insulin has been demonstrated. For example, an abnormal glucose tolerance test and an elevated fasting blood glucose were presumed to be mediated via increased insulin inactivation and/or decreased insulin synthesis or release (Huber and Gershoff, 1973). Gluthathione (GSH) promotes the reductive cleavage of insulin through the glutathione insulin transhydrogenase system working as a hydrogen donor, and is regenerated from oxidized gluthathione (GSSH) by glutathione reductase. If GSH formation is increased in the liver of zinc deficient animals, it may increase the rate of degradation of insulin by the liver. This would result in reduced circulating insulin in zinc deficient animals. Hsu, Anthony, and Buchanan (1967) reported an increased labelled amino acid incorporation into liver glutathione (GSH) in zinc deficient rats.

One of the suggested effects of estrogens on lipid metabolism, is its inhibitory action on lipid catabolic enzyme, lipoprotein lipase (Spellacy, 1973). Whether or not the inhibited enzyme activity appears
in other body tissues other than plasma, is not known. This suppression may be presumed to reduce triglyceride degradation, which, in turn, may save the body fat in zinc deficient rats.

As well as the composition of carcass lipids (Table 10), liver composition (Table 7) shows some influence of diet and steroid treatment. Total liver weight of zinc deficient rats was half of that of zinc supplemented groups. And the lipid contents are a reflection of the weights of livers of the four groups. Steroid injection tended to increase liver weight with an increase of its lipid content. In terms of the percentage of body weight, deficient rats had larger livers than supplemented rats.

Except for the increased serum total cholesterol level (Macapinlac, Pearson and Darby, 1966) in the zinc deficient rats, nothing has been reported in detail about lipid metabolism in zinc deficient animals.

Weight gain with an increased salt and water retention in women has been suggested to be produced by oral contraceptives. However, its precise causes are not shown to be clear. As Beckenhoff, Vetter and Ambruster (1960) reported, when steroid therapy elevates plasma aldosterone level, theoretically, it causes a sodium retention from the tubular lumen. Then, the resulting high osmolality will induce the secretion of antidiuretic hormone, which promotes water conservation. This may result in expansion of total body water compartment. Again, the sodium retaining influence of estrogens is not consistent among women. Liddle (1968) mentioned that large doses of progesterone increased sodium and potassium excretion, and that "the protein wasting process is the wasting of potassium." In this experiment, progesterone
derivatives of Medroxyprogesterone was used in the amount of 0.1 mg, while the suggested dose for the inhibition of gonadotrophin is 0.01 mg for subcutaneous administration (Dorfman, 1969). In this experiment steroid treatment showed a slight decrease and zinc deficient diet caused a significant increase in body moisture content (Table 9). No published data is available about body composition of zinc deficient animals.

Carcass minerals

Most of the body tissues show a reduced zinc level in zinc deficient animals, i.e., a significantly decreased bone zinc content (Prasad et. al., 1967), a decreased hair zinc level, a decreased muscle zinc, etc., (Miller, 1969). Consequently, a decreased total body zinc content would be expected (Table 9). Calcium levels of those tissues which had reduced zinc content, has been increased by zinc deficient diet treatment, the tissues such as muscle, esophagus, liver, but bone (Prasad, 1967). Total carcass ash (expressed in the base of dry and fat free) is increased in zinc deficiency.

Contraceptive steroids has, in general, an anabolic effect on mineral metabolism. Estrogen is said to stimulate osteoblastic proliferation and increase its activity, which leads to Calcium retention (Eisenberg, 1969). The data reported by McBean (1971) agrees with the effect of estrogen reported by Eisenberg. Young female rats receiving contraceptive steroids had heavier femurs with higher Calcium concentration in comparison to controls. Table 9 indicates that zinc deficient diet treatment caused a significant elevation in carcass Calcium
level, and steroids administration resulted in a further increase. In zinc deficient rats, however, its effect appeared much less than in zinc supplemented rats.

It could appear that the phenomenon of increased carcass calcium in zinc deficiency may be due to a greater soft tissue mass in the zinc supplemented animals with its increased potassium and magnesium which may cause an apparent decrease in carcass calcium when expressed on an ash concentration basis.
The 49 female rats were divided into four groups and treated for eight weeks. Twenty-five rats were fed diets containing 2 ppm zinc and 24 rats were fed diets containing 140 ppm zinc. Thirteen rats from the zinc deficient group and 12 rats from the zinc supplemented group were injected daily and intramuscularly with 0.1 ug of Ethynyl Estradiol and 0.1 mg Medroxyprogesterone in 0.1 ml of corn oil, and 12 rats from zinc deficient and zinc supplemented group were treated with placebo injections.

Weekly body weight gain and serum zinc level was measured. The concentrations of carcass moisture, lipids and ash (including carcass zinc and calcium levels) were determined. Liver lipid composition also was determined.

Findings included the following:

1. Zinc deficient diet: It caused significantly low food intake with low growth rate and steep decreased serum zinc level. Zinc supplementation (140 ppm) increased serum zinc over the average normal level.

As zinc deficient rats did not ovulate during this experiment, the ovaries of the rats were much smaller than those of supplemented and contained an increased total cholesterol per mg of tissues. Similarly, zinc deficient diet caused a significant (0.05) increase in total cholesterol content per mg of tissues. The weight of adrenals per 100 gram of body weight was not different compared to that of zinc supplement.
Zinc deficient diet markedly reduced total carcass lipid content with an increase in carcass cholesterol level. This diet caused a significantly (0.05) increased carcass ash with carcass zinc and carcass calcium contents.

2. Oral contraceptive treatment: It tended to increase diet consumption with less weight gain and to decrease serum zinc content. However, they were non-significant.

Contraceptive steroids produced non-functional ovaries with statistically significant decrease in size and increase in total cholesterol content per mg of tissues. This steroid treatment resulted in the almost same state of ovaries, in terms of sizes per 100g of body weight and total cholesterol contents, in zinc supplemented and in zinc deficient rats. The size of adrenals of steroid treated rats was significantly small and they contained increased total cholesterol content.

Total carcass ash percentage, carcass zinc and calcium contents were increased by steroid injections. Contraceptives increased total carcass lipid content in zinc deficient group but decreased the content in zinc supplemented group.

According to the data obtained in the present study, low zinc diet caused a body composition change and defective steroid producing organs. Oral contraceptive steroid showed effective influences in the physiology of ovaries and adrenals and which was also influenced by zinc in their diets.

Therefore, for further work it may be desirable to investigate the total and free cholesterol level of the steroid producing organs being related to zinc contents in serum and in those organs. More thorough
analysis of body composition and an addition of another group of pair fed animals may be recommended.
LITERATURE CITED


APPENDICES
Appendix A

Iodine-Absorption-Number (Hanus Method)

I. Reagent

Preparation of Standard ca 0.1-N-Na$_2$S$_2$O$_3$ Solution

A. 1. Dissolve 25g of Na$_2$S$_2$O$_3$ 5H$_2$O in one liter of water.
2. Boil gently for 5 min.
3. Transfer while hot to storage bottle previously cleaned with hot H$_2$SO$_4$-K$_2$Cr$_2$O$_7$ solution and rinsed with warm boiled water.
4. Store in dark, cool place.
   Note: Do not return unused portion to stock bottle.

B. 1. Weigh 0.2-0.23 g K$_2$Cr$_2$O$_7$ (NBS Standard Sample dried for two hours at 100°C) and place in flask.
2. Dissolve 2gr of KI in 80ml of Cl-free water (boiled and cooled water).
3. Dissolve #1 in #2 solution.
4. Add 20ml of 1-N-HCl with whirling, and immediately place in dark for ten minutes.
5. Titer with Na$_2$S$_2$O$_3$ solution until the red color turns to blue. (red---dark green---blue; about 37ml - 40ml is required).

C. Calculation

Normality of Na$_2$S$_2$O$_3$ solution = \frac{g \cdot K_2Cr_2O_7 \times 1,000}{ml \cdot Na_2S_2O_3 \times 49.032}

Example:

N of Na$_2$S$_2$O$_3$ = \frac{0.20 \times 1,000}{39.5 \times 49.032} = 0.103

.. 15% KI solution
   Dissolve 15 g of KI in 100 g of water.

.. Starch solution
   Dissolve 1 g of starch in cold water, and add 100 ml of boiling water and boil about one minute while stirring.

.. 1-N-HCl
   Pipet about 9.9 ml of 37.7% of stock HCl, and add water to make 100 ml.
II. Procedure of Iodine-Number Determination

1. Weigh 0.5g of fat or 0.25g of oil into 500 ml flask, and dissolve with 10 ml of Choloroform; CHCl₃.

2. Pipet 25 ml of Hanus-Iodine solution, draining pipet definite time.

3. Let stand for 30 minutes in dark, shaking occasionally.

4. Add 10 ml of 15% KI solution, shake thoroughly.

5. Add 100 ml of freshly boiled and cooled water, washing down any free I on stopper.

6. Titr. with 0.1-N-Na₂S₂O₃ adding it gradually and shaking constantly, until yellow turns into colorless. (dark red purple—red—yellow—colorless)

7. Add few drops of starch indicator, which makes solution blue.

8. Continue titration until blue entirely disappears. Toward the end of titr. stopper bottle and shake violently.

9. Calculation

\[
\text{Iodine-Number} = \frac{(\text{ml of Na}_2\text{S}_2\text{O}_3 \text{ used for Blank} - \text{ml of that for Sample})}{\text{g of Sample lipid}} (\text{Normality of Na}_2\text{S}_2\text{O}_3 \times 12.69)
\]

Reference:

APPENDIX B
Appendix B

Method for Fractionation of Three Main Lipid Classes

Silicic acid used as a selective filter which retains one lipid while another is being washed through the column.

About 18g of silicic acid weighed from a batch of adsorbent which has been dried at 115° overnight, is dusted into the glass column; with gentle tapping of the sides of the cylinder as the adsorbent is gradually added, the column adsorbent settled slowly into place with a flat upper surface. Finally, another circular disk of filter paper was placed, without pressure, on the top of the column.

The column was successively washed with 10 ml of ethyl ether, 30 ml of acetone - ethyl ether (1:1, V:V) and finally 20 ml of ethyl ether. When it was completed, the column was attached to the solvent reservoir containing petroleum ether (b.p. 60-70°), and allowed to wash slowly for 10 to 20 hours to assure complete removal of the dehydrating solvents.

A column prepared in this way has the following characteristics: column volume = 22 ml; flow rate of petroleum ether under pressure of the solvent reservoir = 0.3 to 0.6 ml per minute.

When the petroleum ether washing was finished, any solvent remaining above the column was gently aspirated off without allowing the upper surface of the column to dry. About 300 mg of the mixture of lipid sample to be separated was dissolved in 20 ml of petroleum ether, pipetted on to the column. The sides of the glass cylinder above the column was washed with one to two ml of petroleum ether.
All solvents used for elution were redistilled in glass. Ethyl ether, specially, was distilled over sodium and stored at 0-5°C.

Elution was carried out with solvent mixtures of increasing polarity and produced an orderly migration of lipid classes from the column.

Successive solvent mixtures were applied when the last remnants of the preceding mixture disappeared into the column. For the first fractionation of cholesterol ester; 350 ml of 1% ethyl ether in petroleum ether, then 60 ml of 4% ethyl ether in petroleum ether, was applied.

Since care must be taken to prevent the head of the column becoming dry, the timing of these changes of eluants required considerable attention. Therefore, at the time of each solvent change, the low stop cock was closed and the glass tip beneath the stop cock was rinsed with a few ml of chloroform which is added to the eluate last collected.

For the second fractionation of Triglycerides, 300 ml of ethyl ether was applied.

For the third fractionation of phospholipids, 400 ml of absolute methanol was applied.

When, elution is completed by the addition of methanol, ethyl ether (10 ml) was added slowly, 30 ml of acetone-ethyl ether (1:1, V:V) and finally 20 ml of ethyl ether in order to clean the column for the fractionation of next lipid sample.
APPENDIX C
### Vitamin mixture used in the basal diet*

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>g/kg of vitamin mixture</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin A concentrate (200,000 units per gm)</td>
<td>4.5</td>
</tr>
<tr>
<td>Vitamin D concentrate (400,000 units per gm)</td>
<td>0.25</td>
</tr>
<tr>
<td>α-Tocopherol</td>
<td>5.0</td>
</tr>
<tr>
<td>Ascorbic Acid</td>
<td>45.0</td>
</tr>
<tr>
<td>Inositol</td>
<td>5.0</td>
</tr>
<tr>
<td>Choline Chloride</td>
<td>75.0</td>
</tr>
<tr>
<td>Menadione</td>
<td>2.25</td>
</tr>
<tr>
<td>p-Amino Benzoic Acid</td>
<td>5.0</td>
</tr>
<tr>
<td>Niacin</td>
<td>4.5</td>
</tr>
<tr>
<td>Riboflavin</td>
<td>1.0</td>
</tr>
<tr>
<td>Pyridoxine Hydrochloride</td>
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</tr>
<tr>
<td>Thiamine Hydrochloride</td>
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</tr>
<tr>
<td>Calcium Pantothenate</td>
<td>3.0</td>
</tr>
<tr>
<td>Biotin</td>
<td>0.02</td>
</tr>
<tr>
<td>Folic Acid</td>
<td>0.09</td>
</tr>
<tr>
<td>Vitamin B₁₂</td>
<td>0.00135</td>
</tr>
</tbody>
</table>

* Vitamin diet fortification mixture. Nutritional Biochemical corporation. Cleveland, Ohio.