

INVESTIGATIONS ON HYPERVITAMINOSIS E IN RATS

by

IAN BRUCE MACDONALD

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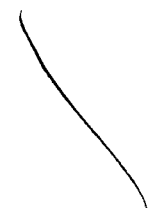
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Department of Human Nutrition

The University of British Columbia  
2075 Wesbrook Place  
Vancouver, Canada  
V6T 1W5

Date April 24, 1979

## ABSTRACT

In view of the fact that some fat soluble vitamins are toxic in large doses to experimental animals and man, this study was initiated to investigate the long-term effects of low, moderate and high levels of dietary vitamin E on various metabolic parameters in the rat.

Six groups of female Wistar rats (50 g) were fed for as long as 16 months the basal vitamin E-free diet with supplements ranging from 0 to 25,000 IU vitamin E (DL- $\alpha$ -tocopheryl acetate) per kilogram diet. The levels of vitamin E chosen were 0, 25, 250, 2,500, 10,000 and 25,000 IU/kg diet; 0 representing vitamin E-free, 25 representing moderate level and 250 to 25,000 representing large doses. All nutrients in the basal diet except vitamin E were adequate.

The focus of this study was on the effects of large doses of dietary vitamin E on: (1) the hematological indices such as hematocrit and hemoglobin levels, prothrombin time and erythrocyte hemolysis at 9, 12 and 16 months of treatment; (2) urinary creatine and creatinine levels at 11 months of treatment; (3) body weight and various organ weights at 8 and 16 months of treatment; (4) femoral parameters such as ash content, and calcium and phosphate concentrations of bone at 8 and 16 months of treatment; and (5) the levels of  $\alpha$ -tocopherol, vitamin A, total lipids, and cholesterol in liver and plasma at 8 and 16 months of treatment.

Rats fed 10,000 and 25,000 IU vitamin E/kg diet for 8 and 16 months had significantly reduced body weights in comparison to those receiving the moderate level of vitamin E. The depressing effect of excess dietary vitamin E on body weight was not as marked as that of vitamin E deficiency. There was little difference between the

moderate and high vitamin E supplemented groups with respect to the weights of liver, uterus and kidney. However, high levels of dietary vitamin E increased the relative heart weights after 8 months and the spleen weights after 16 months.

Hemoglobin and hematocrit values were not influenced by excessive amounts of vitamin E after 9 or 12 months of treatment. At 16 months however, the hematocrit values of rats fed 10,000 and 25,000 IU vitamin E/kg diet were increased significantly over those of rats fed 25 IU/kg diet. The prothrombin time was reduced in rats treated with excess dietary vitamin E for 12 and 16 months. Only vitamin E deficiency, but not excess vitamin E, was associated with increased membrane fragility of erythrocytes.

In rats subjected to excess vitamin E for 16 months the ash content of bone was decreased. High levels of dietary vitamin E increased the plasma alkaline phosphatase activity after 16 months of treatment. These results indicate that there may be increased mineral turnover in bones of rats fed high levels of vitamin E for prolonged periods.

Urinary levels of creatine and creatinine were not affected by high levels of dietary vitamin E. However, in the vitamin E deficient rats, the creatine excretion increased while the creatinine excretion decreased, resulting in a very high ratio of creatine/creatinine in urine.

The  $\alpha$ -tocopherol stored in liver rose significantly with increasing dietary vitamin E. A logarithmic relation was demonstrated between liver  $\alpha$ -tocopherol concentration and dietary levels of vitamin E. The total  $\alpha$ -tocopherol in whole liver of rats fed the different levels of

vitamin E for 16 months was approximately double that in rats treated for 8 months. A curvilinear relationship between plasma tocopherol and the logarithm of dietary vitamin E was found in rats treated for 8 and 16 months.

Total lipids in liver increased significantly with increasing dietary vitamin E in rats treated for 8 months, but not in rats treated for 16 months. There was little difference in liver cholesterol concentration between the moderately supplemented and highly supplemented groups. Increasing dietary vitamin E significantly lowered plasma total lipids and cholesterol in rats treated for 16 months. A quantitative examination of the data showed that the reduction in plasma total lipids was not simply a reflection of the cholesterol levels, and suggests that a high dietary level of vitamin E affected one or more of the constituents of the total lipids (phospholipids and/or triglycerides) other than cholesterol.

From the findings of this long-term study, it appears that high levels of dietary vitamin E result in biochemical changes in some aspects of metabolism in rats. Some of the changes worth recognition are the depression in body weight, increase in relative spleen and heart weights, decrease in ash content of bones with concurrent increase in plasma alkaline phosphatase activity, increased hematocrit value and fatty liver in rats treated for 8 months. A logarithmic relationship was observed between dietary levels of vitamin E and the concentrations of this vitamin in liver and plasma. The results of this study suggest that excess vitamin E over prolonged periods of time have some harmful effects in rats.

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## CHAPTER 1

### INTRODUCTION

Vitamin E has generally been considered to be non-toxic. In recent years there has been considerable interest among the lay public regarding the possible pharmacological role of vitamin E when taken in large dietary supplements ("megavitamin E therapy"). At the present time, there is no satisfactory scientific or clinical evidence to prove that vitamin E supplementation is beneficial for health. In isolated cases, amounts greatly exceeding the normal dietary intake have been administered to human subjects with no significant, adverse clinical effects (Farrell and Bieri, 1975). Nevertheless, it is far from certain that chronic ingestion of vitamin E in megadoses is entirely safe. In human beings, side-effects of excess vitamin E have been reported as fatigue (Briggs et al., 1974), creatinuria (Briggs et al., 1974; Hillman, 1957) and lengthened prothrombin time when taken in excess along with warfarin and clofibrate treatment (Corrigan and Marcus, 1974).

There have been reports of metabolic abnormalities induced in experimental animals by excess vitamin E. March et al. (1973) reported that hypervitaminosis E induced reticulocytosis, lowered hematocrit value, reduced thyroid activity and increased requirements for vitamin D and vitamin K in chicks. Hypervitaminosis E has also been found to depress the activity of glutathione peroxidase in liver and plasma of rats (Yang et al., 1976). Early studies reported that excess vitamin E caused testicular degeneration and reduced fertility

in male rats (Escudero and Herraiz, 1942), and affected the length of estrus cycle and ovarian activity in female rats (Reiss, 1941).

In view of the reports of hypervitaminosis E in experimental animals the purpose of this study was to investigate further the long-term effects of high intakes of dietary vitamin E on rats treated with levels ranging from 0 to 25,000 IU/kg diet. The focus was on the effect of excess intake of vitamin E on the following metabolic parameters: (1) hematocrit and hemoglobin levels, prothrombin time and erythrocyte hemolysis; (2) urinary creatine and creatinine levels; (3) body weight and various organ weights; (4) bone ash content, and calcium and phosphate concentration of bone; and (5) the levels of  $\alpha$ -tocopherol, vitamin A, total lipids and cholesterol in liver and plasma. These parameters were compared statistically with the same parameters in rats receiving a moderate or normal level of dietary vitamin E.

## CHAPTER II

### REVIEW OF LITERATURE

#### A. History of Vitamin E<sup>1</sup>

Evans and Bishop (1922) discovered a fat soluble antisterility factor for the rat, which was designated vitamin E by Sure (1924). Evans proposed to name the substance tocopherol, from the Greek words "tocos" meaning childbirth, "phero" meaning to bring forth and the suffix "ol", it being an alcohol. Much of the pioneer history of vitamin E was reviewed by Evans (1962) and by Mason (1977).

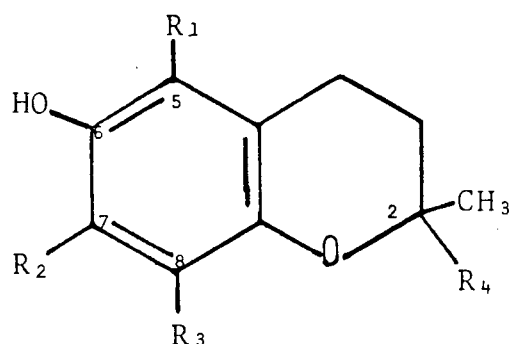
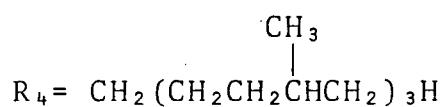
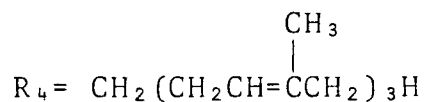
The multiple nature of the vitamin began to unfold in 1936, when Evans et al. (1936) succeeded in isolating from wheat germ oil two compounds with vitamin E activity,  $\alpha$ -tocopherol and  $\beta$ -tocopherol. Since that time, studies of vitamin E have been conducted by numerous investigators (Pennock et al., 1964; Stern et al., 1947). To date, eight structurally similar forms, all derivatives of chromanol-6-ol, have been discovered to have varied amounts of vitamin E activity. The tocopherols belong to two distinct series of compounds, the tocopherols and the tocotrienols. The basic structure and the classification of these compounds accepted by the IUPAC-IUB Commission on Biochemical Nomenclature (1979) are shown in Figure 1. The elucidation of the structure and synthesis of the tocopherols has been reviewed by Sebrell and Harris (1972).

The differences in number and position of the methyl groups affect the biological activity of the various forms of tocopherols. The evaluation of the relative potency of the many compounds which have

---

<sup>1</sup> Definition of terms: The accepted names are vitamin E or tocopherols.

FIGURE 1

STRUCTURE AND NOMENCLATURE OF THE TOCOPHEROLS<sup>1</sup>TocolsTocotrienols

Tocol Structure	Tocotrienol Structure	Methyl Positions
$\alpha$ -tocopherol	$\alpha$ -tocotrienol	5,7,8
$\beta$ -tocopherol	$\beta$ -tocotrienol	5,8
$\gamma$ -tocopherol	$\gamma$ -tocotrienol	7,8
$\delta$ -tocopherol	$\delta$ -tocotrienol	8

<sup>1</sup>IUPAC-IUB Commission on Biochemical Nomenclature (1979) Generic Descriptors and Trivial Names for Vitamins and Related Compounds. J. Nutr. 109, 8-15.

vitamin E activity has been carried out using in vitro tests (Bunyan et al., 1960; Rose and Gyorgy, 1952) and bioassays (Bunyan et al., 1960; Dicks and Matterson, 1962; Friedman et al., 1958; Rose and Gyorgy, 1952). Investigation on the relationship between dosage and response for vitamin E in the fetal resorption test (Bliss and Gyorgy, 1951) led to the acceptance by the National Formulary of the American Pharmaceutical Association (1960) of the conversion factors for the various forms of vitamin E as shown below:

1 mg dl- $\alpha$ -tocopherol acetate	= 1.0 International Unit
1 mg dl- $\alpha$ -tocopherol	= 1.1 International Unit
1 mg d- $\alpha$ -tocopherol acetate	= 1.36 International Unit
1 mg d- $\alpha$ -tocopherol	= 1.49 International Unit

## B. Vitamin E Deficiency - Occurrence

### 1. Human Infants

The clinical evidence of vitamin E deficiency has been seen in the early phase of life, usually with small premature infants. This results from the poor transfer of vitamin E across the placenta, so the infants have low levels of vitamin E in both tissues and blood. The anemia in premature infants is hemolytic in nature and associated with an abnormally elevated erythrocyte fragility by hydrogen peroxide (Bunyan et al., 1960; Rose and Gyorgy, 1952). In treating the anemia, Gross and Melhorn (1972) have found that the absorption of orally administered  $\alpha$ -tocopherol acetate is inefficient in gestationally immature infants and is followed by a favorable hematologic response only when the chronologic equivalent of gestational maturity is reached.

A state of vitamin E deficiency occurs in individuals who have



a defect in their ability to absorb fat (Binder and Shapiro, 1967; Machon and Neals, 1970; Muller and Harris, 1969). A large number of these cases are in children and young adults with cystic fibrosis (Bieri and Farrell, 1976). Lower than usual blood tocopherol levels are observed in diseases where intestinal absorption is affected, but no symptomatology which responds to vitamin E has been observed. A thorough review of the information available on vitamin E status in other malabsorptive states has been conducted by Bieri and Farrell (1976).

## 2. Human Adults

There are no reported clinical evidences of a deficiency of vitamin E in normal human adults because of the considerable tissue storage of the vitamin and the consequent extended period required for depletion (Bieri, 1975). It has been suggested that serum tocopherol levels below 0.5 mg/100 ml could be classified as deficient (Bieri and Farrell, 1976). Some investigators have shown that there is a tendency for serum  $\alpha$ -tocopherol to rise and fall in proportion to the amounts of cholesterol, phospholipid and triglycerides present in the blood (Davies et al., 1969; Horwitt et al., 1972). Hence, interpretation of the status of vitamin E nutriture from blood data may not accurately reflect either the level of intake or tissue storage. Furthermore, since blood tocopherol is only about 1 per cent of the total body tocopherol pool, it is sometimes difficult to relate blood tocopherol to vitamin E nutriture.

A long-term study by the Food and Nutrition Board of the National Research Council (Horwitt, 1962) was carried out by feeding a partially deficient vitamin E diet to men for 5 years. There were no obvious clinical signs of vitamin E deficiency in these subjects even though the

blood tocopherol levels fell up to 0.3 mg/100 ml. The half-life of the erythrocytes was decreased, but there were no obvious manifestations of anemia.

### 3. Animals

Vitamin E deficiency can be demonstrated in animals fed diets low in vitamin E. There are a number of vitamin E deficiency states in different species of animals, but skeletal muscle is the most universally affected tissue. Some of the signs of vitamin E deficiency in different species of animals are shown in Table 1. A thorough documentation of the vitamin E deficiency states in animals are reviewed by Green (1972a) and Scott (1970).

### C. Cellular Function of Vitamin E

A full understanding of the mode of action of vitamin E at the molecular level has not yet been reached. With the several different, apparently unrelated disease states in different animal species arising from vitamin E deficiency it has been difficult to determine a basic role for the vitamin in cellular metabolism. There are two major interpretations put forth by investigators to explain the mechanism of action of vitamin E, the biological antioxidant theory and the specific metabolic function theory.

The biological function of vitamin E as a lipid antioxidant has been investigated for nearly forty years, since Olcott and Mattel (1941) discovered the antioxidant activity of vitamin E. The biological antioxidant theory suggests that tissue unsaturated lipids are constantly under attack by free radicals and that in the presence of oxygen they become peroxidized. If sufficient vitamin E is not present, the

TABLE 1

Syndromes Resulting From Vitamin E Deficiency<sup>1</sup>

Animal Species	Syndrome
Rat (male) (female) (both sexes)	sterility fetal resorption liver necrosis
Rabbit	muscular dystrophy myocardial degeneration
Dog and guinea pig	myocardial degeneration
Chicken <sup>8</sup>	encephalomalacia exudative diathesis
Primate	macrocytic anemia muscular dystrophy

<sup>1</sup> From Nair, 1972.

peroxidation of lipids becomes extensive and uncontrolled, leading to widespread damage to intracellular membranes, enzymes and certain metabolites such as vitamin A and phospholipids. All the diverse effects of vitamin E deficiency in animals are considered to be secondary, stemming from one primary process, lipid peroxidation. Scientific evidence attempting to show that vitamin E functions as a lipid antioxidant has been presented in numerous reviews (Tappel, 1962; Tappel, 1972; Witting, 1970).

In recent years there has been considerable research which has exposed major weaknesses in the antioxidant theory of vitamin E (Bunyan et al., 1968; Green and Bunyan, 1969; Green, 1972b). Controversy has developed as to whether or not lipid peroxidation occurs during vitamin E deficiency. There is no doubt that vitamin E has antioxidant properties which can inhibit tissue lipid peroxidation in vitro. Several investigators have argued that lipid peroxidation does not occur in vivo and therefore the biological function of the vitamin must be unrelated to its antioxidant activity. However, a recent experiment by Hafeman and Hoekstra (1977) indicated that lipid peroxidation occurs in vivo in rats as a result of vitamin E deficiency and the peroxidation process is greatly accelerated during the terminal phase of the fatal condition. This report opposes the basic argument of the critics of the antioxidant theory. Much more research, however, is needed to strengthen the antioxidant hypothesis.

Several investigators have recently postulated that vitamin E may act as a catalyst or regulatory agent in intermediary metabolism, at a specific site which is of fundamental importance in metabolism (Green, 1972b; Nair, 1972; Schwarz, 1972). In spite of the huge amount of data

on vitamin E published in the literature, an unequivocal, direct involvement of vitamin E in specific metabolic functions has yet to be identified. It is known that the activity of many enzyme systems are altered in vitamin E deficient animals (Green, 1972a; Mason and Horwitt, 1972a). Whether the alteration of enzymatic activity is primary or secondary to the breakdown of other tissue components is still a controversy.

Research has been stimulated at the molecular level for a direct involvement of vitamin E in many enzyme functions. Muscle creatine kinase (Olson, 1974), liver microsomal enzyme drug hydroxylating complex (Carpenter and Howard, 1974), liver xanthine oxidase (Catignani et al., 1974), bone marrow  $\gamma$ -aminolevulonic acid synthetase and liver  $\gamma$ -aminolevulonic acid dehydratase (Caasi et al., 1972; Nair, 1972) are some of the enzymes that have received most attention. These studies on rates of enzyme synthesis suggest a role for vitamin E in the regulation of protein synthesis. Just how vitamin E may possibly participate in this sequence of events is not known. This area has been thoroughly reviewed by Molenaar et al. (1972) and Bieri and Farrell (1976).

At this time there is no definitive evidence to explain many of the biochemical derangements evoked by a deficiency of vitamin E in animals. Investigators at this time must consider that both the biological antioxidant theory and the specific metabolic function theory of vitamin E action are viable and that they may be neither inconsistent nor mutually exclusive.

#### D. Pharmacological Effects of Vitamin E in Human Subjects

Even though it seems unlikely that a natural deficiency of vitamin E occurs in man there is good reason to believe that a large

segment of the North American population is consuming supplementary doses of vitamin E (Farrell and Bieri, 1975). Much of the popular interest in vitamin E stems from articles in magazines, books and newspapers dealing with the therapeutic efficacy of vitamin E for disorders ranging from cardiovascular disease to muscular dystrophy. A critical appraisal of the therapeutic value of vitamin E has been made by Marks (1962) and Bieri and Farrell (1976). Tocopherol supplements either self-administered or prescribed by physicians vary widely in dosage, but average to about 400 IU vitamin E/day.

Vitamin E has been presumed to be nontoxic to human and animals (Briggs and Briggs, 1974; Farrell and Bieri, 1975; Horwitt and Mason, 1972). While the undesirable side effects have been rarely reported, it is difficult to evaluate what possible pharmacological action results from the ingestion of vitamin E at many times the generally recognized nutritional requirement. Very few critical studies of megavitamin E supplementation in man have ever been carried out.

In the only systematic investigation of megavitamin E supplementation in human, Farrell and Bieri (1975), gave 100 to 800 IU vitamin E/day for 3 years to 28 adults. Laboratory screening for toxic side effects of vitamin E supplementation by clinical blood tests failed to reveal any disturbance in liver, kidney, muscle, thyroid gland, erythrocytes, leucocytes, coagulation parameters or blood glucose. It was concluded that megavitamin E supplements in this group produced no toxic side effects.

Beckman (1955) has reported that vitamin E was given to patients for months, both orally and parenterally at a dosage level of 300 IU vitamin E/day without any adverse clinical effects. Greenblatt (1957) supplemented

the diet of six men with a massive dose, 40 g d- $\alpha$ -tocopherol acetate/day for one month. There were no adverse clinical signs reported. However, Hillman (1957) reported that ingestion of 2 to 4 g vitamin E/day by an individual for 3 months produced creatinuria, cheilosis, angular stomatitis, gastrointestinal disturbance and muscular weakness. These toxic side effects ceased within two weeks after the vitamin E supplementation was discontinued. Vogelsang et al. (1947) reported that vitamin E supplementation resulted in hypoglycemia and depressed prothrombin levels, the latter suggesting a relative vitamin K deficiency.

The involvement of vitamin E in potentiating some anti-coagulation activity has been reported in two studies. Korsan-Bengtson et al. (1974) reported prolonged plasma clotting time in 9 subjects receiving 300 IU  $\alpha$ -tocopherol/day. Corrigan and Marcus (1974) observed a prolonged prothrombin time in a patient ingesting 800 IU vitamin E/day, plus warfarin and clofibrate. A reduction of the level of vitamin K-dependent coagulation factors was noted during the period of vitamin E ingestion, which returned to base-line levels after the patient stopped taking the vitamin E.

An examination of the mechanism by which vitamin E might be antagonistic to vitamin K-dependent clotting activity has led to an evaluation of the biological metabolites of the tocopherols. Woolley (1945) reported that  $\alpha$ -tocopherylquinone was an antimetabolite of vitamin K<sub>1</sub>. This structural analog of vitamin K<sub>1</sub> was reported to cause hemorrhages in the reproductive systems of pregnant mice. The action of the  $\alpha$ -tocopherylquinone was prevented by small amounts of vitamin K<sub>1</sub>. Subsequent research by March et al. (1973) with chickens, and Rao and Mason (1975) with rats, offers further evidence that metabolites of the tocopherols may serve as

competitive inhibitors of vitamin K<sub>1</sub>.

## E. Pharmacological Effects of Vitamin E in Animals

### 1. Growth

The findings on the effect of excess dietary vitamin E on the growth rate in animals vary widely. March et al. (1973) found a depressed growth rate in chicks fed a 2,200 IU vitamin E/kg diet from hatching to 50 days. Growth rate was not seen to be affected by supplementation of 1,000 IU vitamin E/kg diet. Nockels et al. (1975) reported that feeding chicks 2,000 or 4,000 IU vitamin E/kg diet for 5 weeks had no significant effect on body weight. However, higher levels of vitamin E supplementation, such as 8,000 and 64,000 IU/kg diet, were reported to reduce the chick body weight significantly.

McCuaig and Motzok (1970) fed a 10,000 IU vitamin E/kg diet to chicks and found the growth rate was unaffected by the supplementary vitamin E treatment. Similar results have also been reported in the rabbit (Awad and Gilbreath, 1975) and the rat (Alfin-Slater et al., 1972) fed excess vitamin E.

However, Jenkins and Mitchell (1975) found that the growth rate was increased when rats were fed either a 600 or 6,000 IU vitamin E/kg diet for 2 months.

### 2. Hematology

March et al. (1973) examined reticulocytosis in response to various dietary-antioxidants in chicks. They found that supplementation of either 120 or 220 IU vitamin E/kg diet induced reticulocytosis. At these levels of vitamin E supplementation hematocrit levels were not affected. In a later study, treatment of chicks with larger doses of vitamin E



(2,200 IU/kg diet) was noted to induce both reticulocytosis and a reduction in hematocrit values (March et al., 1973). Jenkins and Mitchell (1975) fed 600 or 6,000 IU vitamin E/kg diet to rats and found no significant effect on the hemoglobin levels.

A significant lengthening of the prothrombin time was observed by March et al. (1973) when a 2,200 IU vitamin E/kg diet was fed to chicks. The lengthened prothrombin time was rapidly normalized by injection with menaquinone. An earlier study by Melette and Leone (1960) was the first to observe that vitamin E supplementation may prolong prothrombin time in rats fed nonirradiated as well as irradiated beef in the diet.

The mechanism by which hypervitaminosis E affects prothrombin time has not yet been fully elucidated. The observation by March et al. (1973) that an injection of vitamin K reversed the lengthened prothrombin time led them to speculate that a metabolite of vitamin E may be a structural analogue of vitamin K. One such compound has been identified in the liver,  $\alpha$ -tocopherol- $\rho$ -quinone (Csallany et al., 1962). An earlier study by Woolley (1945) found that administration of  $\alpha$ -tocopherylquinone to pregnant mice caused hemorrhage in the reproductive system. The action of quinone was prevented by small amounts of vitamin K<sub>1</sub>, but not by large doses of dl- $\alpha$ -tocopheryl acetate. Sufficient amounts of  $\alpha$ -tocopherylquinone may be produced following excessive intake of vitamin E to increase the dietary requirement for vitamin K<sub>1</sub>.

It has been well established that vitamin E deficiency is characterized by spontaneous hemolysis of the erythrocyte or extensive in vitro hemolysis induced by hydrogen peroxide or dialuric acid. Low erythrocyte hemolysis in vitro though does not clearly indicate adequacy of tissue vitamin E stores (Bieri and Poukka, 1970). The existence of variables

other than vitamin E intake that can affect in vitro hemolysis has added to the uncertainty of this test (Macdougall, 1972; Melhorn et al., 1971; Stocks and Dormandy, 1971a; Stocks et al., 1971b). Stocks and Dormandy (1971a) illustrated that peroxide induced erythrocyte autoxidation was influenced by a number of substances such as albumin, plasma and ascorbic acid. Melhorn et al. (1971) have shown that hydrogen peroxide hemolysis of greater than 20 per cent can occur in a wide variety of hematological disorders in which vitamin E concentration is normal. Some of these hematological disorders are: hereditary and acquired anemias, iron deficiency anemia, and hemoglobinopathies.

Hypervitaminosis E has been found to change the fatty acid pattern of erythrocytes (Alfin-Slater et al., 1972). Whether excessively high doses of vitamin E will alter the stability of the erythrocyte membrane has not yet been determined.

### 3. Bone Calcification

Excess amounts of vitamin E were found to depress bone calcification in chicks fed either calcium-deficient or vitamin D-deficient diets. March et al. (1973) found that the adverse effect of hypervitaminosis E on bone calcification was overcome when vitamin D was fed at over 300 IU/kg diet. The mechanism by which the excess dietary vitamin E increased the requirement of vitamin D for maximum bone calcification is not presently known.

### 4. Endocrine Function

Several studies have shown altered endocrine function in experimental animals due to excessive dietary vitamin E intake. The endocrine organs

reported to be affected are the sexual organs (Czyba, 1966a; Escudero and Herraiz, 1942; Masson, 1941; Reiss, 1941), adrenal gland (Hill and Hamed, 1970; Forni et al., 1955; Jenkins and Mitchell, 1975), thymus (Forni et al., 1955); and the thyroid gland (Czyba et al., 1966b; Huter, 1947; March et al., 1973; Valenti and Bottarelli, 1965). While there is little conclusive evidence of any adverse effect of vitamin E on the former three organs, the effect on the thyroid gland is fairly well established.

An early investigator of hypervitaminosis E in female rats observed a hypertrophy of the ovary and alteration in the length of estrus cycle (Reiss, 1941). Other studies found that excess dietary vitamin E reduced male fertility in rats (Escudero and Herraiz, 1942) and hamsters (Czyba, 1966a). However, Masson (1941) reported that feeding excessive amounts of vitamin E to hens had no effect on the birds' fertility.

The evidence of adverse effects of hypervitaminosis E on adrenal function is contradictory. Forni et al. (1955) found that excess vitamin E caused an increase in adrenal weight in rats. In contrast, Jenkins and Mitchell (1975) fed a diet containing up to 6,000 IU vitamin E/kg for eight weeks and reported a significant decrease in adrenal weight. Hill et al. (1960) observed that hypervitaminosis E caused adrenal degeneration.

The only reported effect of hypervitaminosis E on the thymus was made by Forni et al. (1955), who observed a decrease in thymus weight.

Hypervitaminosis E has been reported to have an adverse effect on the thyroid gland. Huter (1947) was the first to report an injury to the thyroid gland in rabbits caused by excess dietary vitamin E. Valenti and Bottarelli (1965) found that hypervitaminosis E reduced thyroid activity in the rat. Czyba et al. (1966) reported that the administration of

vitamin E caused a transitory stimulation of thyroid activity, which was followed by a depression of thyroid function. March et al. (1973) fed a 220 IU vitamin E/kg diet to chicks and assessed the thyroid activity by measuring the rate of uptake and release of  $I^{131}$  by the thyroid gland. They found that the activity of the thyroid was significantly suppressed in response to excess vitamin E. It would be expected that a decrease in thyroid activity would be accompanied by some decrease in growth rate. This was not seen though at this level of vitamin E supplementation, but feeding a ten-fold greater amount of vitamin E (2,200 IU/kg) caused a decreased growth rate.

#### 5. Tissue Storage of Vitamin E

The major pathway of vitamin E absorption from the intestine parallels fat absorption (Pomeranze and Lucarello, 1953). Following absorption, the tocopherol is transported, via the lymphatics, in the chylomicrons (Blomstrand and Forsgren, 1968). Gloor et al. (1966) have shown that  $\gamma$ -tocopherol was absorbed from the intestine almost as efficiently as was  $\alpha$ -tocopherol. Since  $\gamma$ -tocopherol is the predominant tocopherol in the North American diet, calculations based on only  $\alpha$ -tocopherol significantly underestimate vitamin E intakes (Bieri and Poukka Evarts, 1973).

There have been numerous studies attempting to determine the quantitative relationship of increasingly higher levels of vitamin E intake versus plasma and liver tocopherol levels. Losowsky et al. (1972) have examined the efficiency of absorption of dietary tocopherol in both man and animals. Over a narrow dietary range of intake the percentage absorption falls off as the dose is increased. Excretion measurements with rats

indicated a marked decrease in tocopherol absorption efficiency as the dose was increased from the microgram to milligram range (Losowsky et al., 1972).

Bolliger and Bolliger-Quaife (1956) in experiments with rats have reported that the relationship between the dose of tocopherol and its storage in liver is linear when both are expressed as logarithms. They also suggested a linear relationship between plasma tocopherol level and the log of the dose of vitamin E intake. In experiments with the chick, Wiss et al. (1962) reported similar results as the former study. Bieri (1972) also reported a linear relationship between plasma tocopherol concentration and the log of the dietary vitamin E in experiments with rats. Gray (1960) disagreed that such a relationship existed.

In a 28 week study of high vitamin E intake in rats, Alfin-Slater et al. (1972) found that the plasma tocopherol levels reflected the dietary vitamin E intake. The plasma levels were not proportional to the dose administered. Also tocopherol levels in females were almost two-times greater than in male rats. Awad et al. (1975) in a 4 week study with rabbits reported that supplementation with 5,000 IU vitamin E/kg diet increased the plasma and liver tocopherol levels, but only the latter was significantly increased.

These results suggest that the relationship between the dietary level of vitamin E and tissue storage may be variable, depending on the animal species, growth rate of the animal, length of the test period, dosage level and the tissue being examined.

## 6. Tissue Storage of Vitamin A

It has been recognized for many years that there is a nutritional relationship between vitamin E and vitamin A. Many investigators have

found a "sparing" effect of vitamin E on vitamin A. Moore et al. (1940) originally reported that vitamin E increased liver storage of vitamin A in rats over a period of 8 to 12 months. Hichman et al. (1944) confirmed this finding. Other early workers though found no "sparing" effect of vitamin E in experiments limited to 4 weeks (Lemley, 1947; Herbert and Morgan, 1953). The contradictory evidence demonstrated that there was not a simple relationship between the two vitamins, but instead a complex effect, dependent on diet, the dosage regimen of the two vitamins and the length of the experiment.

In spite of the contradictory results reported in the early studies, more recent investigations indicated that dietary vitamin E increases tissue levels of vitamin A (Cawthorne et al., 1968; Jenkins and Mitchell, 1975; Prodouz and Navari, 1975; Roels et al., 1964). Prodouz and Navari (1975) chose dietary levels of vitamin E ranging from 0.00 IU/week to 3.5 IU/week and examined the effect of vitamin E on vitamin A storage in rats. They found a much larger increase in liver vitamin A per IU vitamin E fed than per IU of vitamin A in the diet. In examining the depletion of liver stores of vitamin A Cawthorne and colleagues (1968) reported that supplementary vitamin E significantly decreased the rate of depletion of vitamin A reserves in the rat, thus confirming the results of Moore et al. (1940). This vitamin E effect was shown at remarkable low intakes; even 1 mg was sufficient to produce a three-fold difference on vitamin A storage within 6 weeks in rats. This effect was demonstrable though only when the initial reserves were high, about 30,000 IU vitamin A per liver. The same effect was not observed when the initial liver reserves of vitamin A were only 3,000 IU, which suggests a role for vitamin E in altering the capacity of the liver to bind vitamin A.

The effect of supplementation with high levels of vitamin E on tissue vitamin A storage has been examined by two groups. Roels et al. (1964) reported that a ten-fold increase in dietary vitamin E intake (50 to 500 IU/kg diet) resulted in a 11 percent increase in liver vitamin A storage. In examining the effect of supplementation with 600 or 6,000 IU vitamin E/kg diet Jenkins and Mitchell (1975) confirmed that vitamin E increased the storage of vitamin A in the liver. They also found that the plasma vitamin A was significantly increased when high levels of vitamin E were fed.

Green and Bunyan (1969) suggested that vitamin E may "spare" vitamin A by protection from oxidation in the gut, by increasing vitamin A absorption, by increasing vitamin A efficiency, and/or by increasing the storage of vitamin A. They noted that the antioxidant properties of vitamin E may or may not be significant in the mechanism. Roel et al. (1964) and Jenkins and Mitchell's (1975) findings cannot be explained by the antioxidant effect of vitamin E. Even the supplementary level in the experiment of Roel et al. (1964), 50 IU vitamin E/kg diet was more than adequate for the rats needs, yet excessively larger doses of vitamin E accentuated the "sparing" effect of vitamin E on vitamin A. This supports the proposal of Tappel (1973), DiLuzio (1973) and Green (1972b) that vitamin E may have a more specific in vivo biochemical role in addition to its suggested in vivo and/or in vitro antioxidant properties.

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X

#### 7. Liver Lipid Levels

According to Alfin-Slater et al. (1972) liver cholesterol and total lipid levels increased progressively as the dietary vitamin E intake was

increased. This effect was observed in rats fed high levels of vitamin E for a 28-week period.

Other workers have examined the simultaneous effects of various levels of vitamin E and vitamin A (Harrill et al., 1965; Jenkins and Mitchell, 1975; Prodouz and Navari, 1975), or vitamin E and arginine or methionine (Harrill and Gifford, 1966) on levels of liver cholesterol and total lipids.

Contrary to the results of Alfin-Slater et al. (1972), Harrill and Gifford (1966) found that increasing the dietary level of vitamin E decreased the level of cholesterol and total lipids in rat liver. These findings are not consistently seen though when examining the simultaneous effects of vitamin E and A on tissue lipid levels. Prodouz and Navari (1975) and Harrill et al. (1965) reported that increasing dietary vitamin E significantly decreased liver total lipids and increased liver cholesterol. However, Jenkins and Mitchell (1975) reported that increasing dietary vitamin E significantly increased total lipids and decreased cholesterol in rat liver. The reason for the discrepancy in results in this area remains obscure. It might well be that the ratio of vitamin E to vitamin A is the decisive factor determining the effect of these vitamins on tissue lipid levels in these experiments.

#### 8. Blood Lipid Levels

The relationship between high dietary vitamin E and plasma lipid level is not yet clear. Most investigations in this area have examined the ability of supplemental vitamin E to alter plasma cholesterol levels. Some studies have reported a decrease in serum cholesterol in rats fed vitamin E supplemented diets (Chen et al., 1972; Harrill et al., 1965;



Prodouz and Navari, 1975). Chen et al. (1972) showed that raising the dietary vitamin E intake resulted in lower serum cholesterol levels, proportional to the amount supplemented. The regression curves of cholesterol level to vitamin E intake (up to 50 IU/kg diet) were not linear though.

However, several workers have reported that high dietary vitamin E intakes had no effect on serum cholesterol levels in rabbits (Awad and Gilbreath, 1975; Horn et al., 1962), chicks (Koyangi et al., 1966), and rats (Jenkins and Mitchell, 1975). Awad and Gilbreath (1975) found that diets formulated to contain 5,000 IU vitamin E/kg diet had no effect on serum cholesterol in rabbits. Jenkins and Mitchell (1975) also fed high levels of vitamin E (6,000 IU/kg diet) to rats and observed that plasma cholesterol levels were not significantly affected.

Some investigators have reported that high doses of vitamin E actually caused hypercholesterolemia (Bruger, 1945; Campbell, 1952). Little evidence of the effect of large doses of vitamin E on blood lipid levels can be gained though from either of these two studies because of the unnatural experimental conditions employed. Both studies fed atherosclerotic diets to rabbits and vitamin E was injected intramuscularly.

The level of vitamin E supplementation, the length of treatment and the dietary ingredients vary widely in the experiments reported above. Whether one or more of these conditions can account for the wide diversity of findings reported in the literature is not yet known.

### CHAPTER III

#### MATERIALS AND METHODS

##### A. Animal Care

Ninety female weanling Wistar rats, 45-55 g in weight, were obtained from Biobreeding Laboratories, Ottawa, Ontario. Upon arrival they were randomly divided into six groups of fifteen animals each. For the initial two-week period they were housed in pairs, after which they were housed singly in screen-bottomed stainless steel cages kept in an air-conditioned room maintained at 23-25°C. Lighting was regulated automatically to provide alternate 12-hour periods of light and darkness (light on from 6:00 a.m. to 6:00 p.m.). Food and water were given ad libitum throughout the experimental period of sixteen months (December 1973 to April 1975).

##### B. Experimental Diets

Six experimental diets were used: a tocopherol-free diet, and the same diet supplemented with either 25, 250, 2,500, 10,000 or 25,000 IU vitamin E (dl- $\alpha$ -tocopherol acetate) per kg diet. These were based on a modified Draper's (1964) Standard Vitamin E-Free diet. The composition of the diets and that of the mineral and vitamin mixes used are shown in Table 2. Dietary ingredients were obtained from Texlab Mills, Madison, Wisconsin, U.S.A.

##### C. Experimental Groups

The six experimental groups were designated as shown below.

- Group A : Vitamin E-free diet - Basal
- Group B : Basal diet plus 25 IU vitamin E/kg
- Group C : Basal diet plus 250 IU vitamin E/kg
- Group D : Basal diet plus 2,500 IU vitamin E/kg

TABLE 2

Composition of the Basal Diet<sup>1</sup>

Ingredient	%
D-Dextrose	64.9
Vitamin-free casein	20.0
Corn oil, tocopherol stripped	10.0
Salt mix (no. 4164) <sup>2</sup>	4.0
Vitamin mix <sup>3</sup>	0.6
Choline chloride	0.5

<sup>1</sup>Modified from Draper, H.H., et al. (1964) J. Nutr. 84, 395-400.

<sup>2</sup>Provided the following as g/kg diet:  $\text{CaCO}_3$ , 6.54;  $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$ , 14.2;  $\text{NaCl}$ , 4.3;  $\text{K}_2\text{HPO}_4$ , 3.09;  $\text{K}_3(\text{C}_6\text{H}_5\text{O}_7) \cdot \text{H}_2\text{O}$ , 9.46;  $\text{MgCO}_3$ , 1.64;  $\text{Fe}(\text{C}_6\text{H}_5\text{O}_7) \cdot 3\text{H}_2\text{O}$ , 0.64;  $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ , 0.055;  $\text{ZnCO}_3$ , 0.018;  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ , 0.007;  $\text{KI}$ , 0.0018.

<sup>3</sup>Provided the following amount per kg diet (in IU): 25,000 vitamin A as retinyl palmitate; 2,000 ergocalciferol; (in mg): menadione, 1; biotin, 0.1; vitamin  $\text{B}_{12}$ , 0.1; calcium pantothenate, 10; folic acid, 1; niacin, 25; pyridoxine HCl, 5.0; riboflavin, 5.0; thiamine HCl, 10.

Group E : Basal diet plus 10,000 IU vitamin E/kg

Group F : Basal diet plus 25,000 IU vitamin E/kg

#### D. Experimental Procedures

The experiment was continued over a sixteen month period during which animals were randomly chosen from each group and the following protocol was carried out at predetermined times.

The hematological indices were measured at 9,12 and 16 months of treatment. Blood samples were taken by tail cutting after anesthetizing the rats with anhydrous diethyl ether (Fisher Scientific) for the determination of hemoglobin, hematocrit and erythrocyte hemolysis. Blood was directly drawn from the tail into a sodium oxalate coated Miale prothrombin pipet for estimation of prothrombin time.

At 11 months, a 24-hour urine sample was collected for the determination of urinary creatine and creatinine. The samples were stored in plastic bottles without preservative at  $-20^{\circ}\text{C}$  until analysis.

Four rats from each group were killed after 8 months and the others were killed at the end of 16 months of dietary treatment. The animals were first weighed and then lightly anesthetized with anhydrous diethyl ether. Blood was drawn from the inferior vena cava using a heparinized syringe. Plasma was obtained by centrifugation and placed into small plastic tubes and frozen at  $-20^{\circ}\text{C}$  until further processing. The storage of the individual plasma aliquots permitted avoidance of repeated thawing and re-freezing. Plasma samples were analyzed for vitamin E, cholesterol and total lipids. Plasma alkaline phosphatase activity and vitamin A (retinol) were also measured in rats treated for 16 months.

Immediately after exsanguination, the liver, spleen, heart, kidney and uterus were rapidly removed. They were trimmed for extraneous

tissues, washed in cold physiological saline solution and then weighed. The liver was frozen at  $-20^{\circ}\text{C}$  for analysis of vitamin A, vitamin E, total lipids and cholesterol.

The left femur was removed and stripped of soft tissue. It was then frozen at  $-20^{\circ}\text{C}$  for analysis of bone ash, calcium and inorganic phosphate.

## E. Biochemical Determinations

### 1. Hemoglobin and Hematocrit

Hemoglobin was determined by the spectrophotometric method described by Eilers (1967). A 0.02 ml aliquot of blood was diluted with 5 ml of cyanmethemoglobin reagent (Hyland Division, Travenol Laboratories Inc., Casta Mesa, Calif., U.S.A.) and then read at 540 nm using the Beckman DU-2 spectrophotometer. Hemoglobin concentration was calculated by multiplying optical density (OD) at 540 nm with a factor determined on the hemoglobin calibration curve as shown in Figure 2.

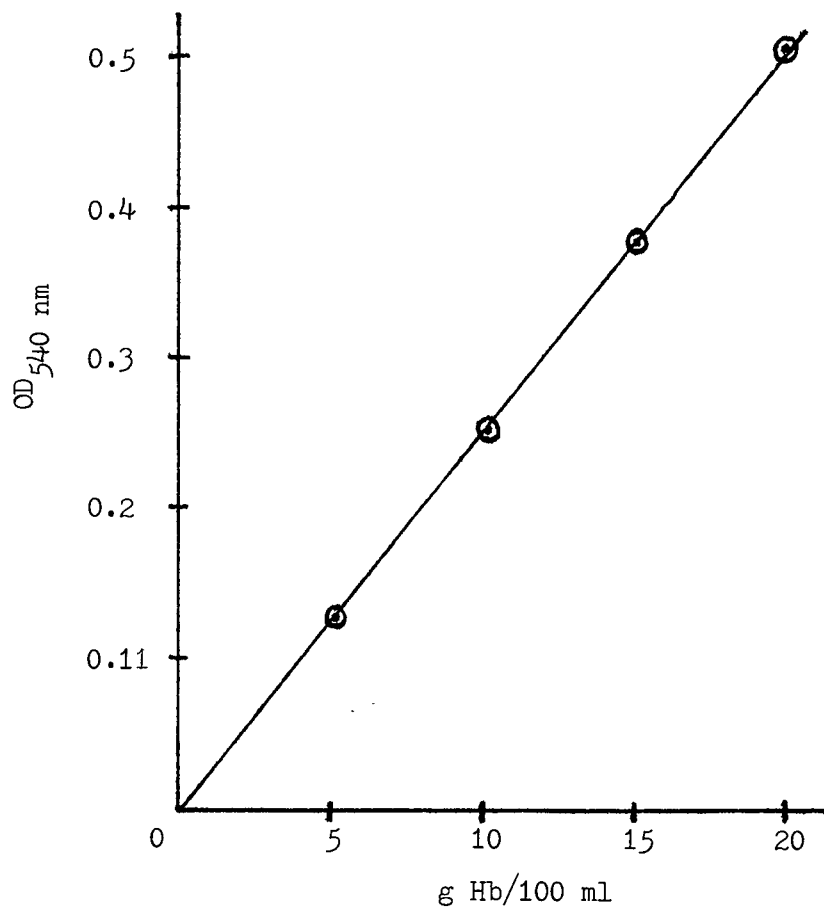
Hematocrit was read from a heparinized micro-hematocrit tube (Fisher Scientific) after centrifugation at  $11,500 \times G$  for 5 minutes (Eilers, 1967).

### 2. Prothrombin Time

The prothrombin time was determined by a micromethod of the standard one-stage prothrombin time method described by Miale and Winningham (1967). This procedure used a siliconized Miale Prothrombin Pipet (Dade, Miami, Fl.) for the collection of capillary blood. It was mixed with a measured amount of sodium oxalate solution (100 mM) and centrifuged to obtain oxalated plasma. The test was then performed by blowing the oxalated plasma into a test tube of thromboplastin- $\text{CaCl}_2$  mixture (Dade) at  $37^{\circ}\text{C}$  and the clotting time was noted.

FIGURE 2

Standard Curve For Hemoglobin



### 3. Erythrocyte Hemolysis

The hemolysis procedure was that described by Draper and Csallany (1966). It is based on the degree of spontaneous hemolysis of erythrocytes in a buffered isotonic saline solution. Following incubation of the erythrocyte aliquots, the absorbance of the supernatants were read at 415 nm on the Beckman DU-2 spectrophotometer.

Therper cent hemolysis was calculated from the formula shown below.

$$\% \text{ hemolysis} = \frac{A_b}{A_c} \times 100$$

where

$A_b$  = absorbance of buffer solution at 415 nm

$A_c$  = absorbance of  $H_2O$  solution at 415 nm

### 4. Urinary Creatine and Creatinine

The urinary creatine and creatinine levels were determined by a method based on the Jaffe reaction as described by Henry et al. (1974).

Creatinine was determined by quantitating the red pigment, alkaline creatinine picrate. The optical density was measured with a Beckman DU-2 spectrophotometer at 500 nm.

The urinary creatinine level was calculated by the following formula.

$$\text{mg creatinine/ml urine} = \frac{A_x}{A_s}$$

where

$A_x$  = absorbance of unknown at 500 nm

$A_s$  = absorbance of standard at 500 nm

The urinary creatinine of the rats was then expressed as follows.

mg creatinine/kg body weight/24 hours

The urinary creatine level was determined by the difference in creatinine before and after the dehydration of creatine to creatinine. The urinary creatine level was then calculated as follows.

$$\begin{array}{l} \text{total creatinine (mg reformed creatinine} \\ \text{plus mg creatine as creatinine/ml urine)} \end{array} = \frac{A_x}{A_s}$$

$$\text{mg creatine as creatinine/ml urine} = \text{total creatinine} - \text{preformed creatinine}$$

The urinary creatine of the rats was then expressed as follows.

$$\text{mg creatine/kg body weight/24 hours}$$

#### 5. Plasma Vitamin A

Plasma vitamin A levels were determined according to the method described by Neeld and Pearson (1963), which is a modification of the classic Carr-Price technique. The blue chromophore produced by the interaction of trifluoroacetic acid and vitamin A in chloroform was measured at 620 nm on a Beckman DU-2 spectrophotometer and gave an indication of the amount of vitamin A present in the plasma.

Standard curves for mg vitamin A/100 ml plasma were established using all trans retinyl acetate (Hoffmann-La Roche Inc., Nutley, N.J., U.S.A.). The average slope of the curve at 620 nm was found to be 7.53. An illustration of this curve is shown in Figure 3.

Plasma vitamin A levels were calculated from the standard curve (Fig. 3) and expressed as µg per 100 ml of plasma.

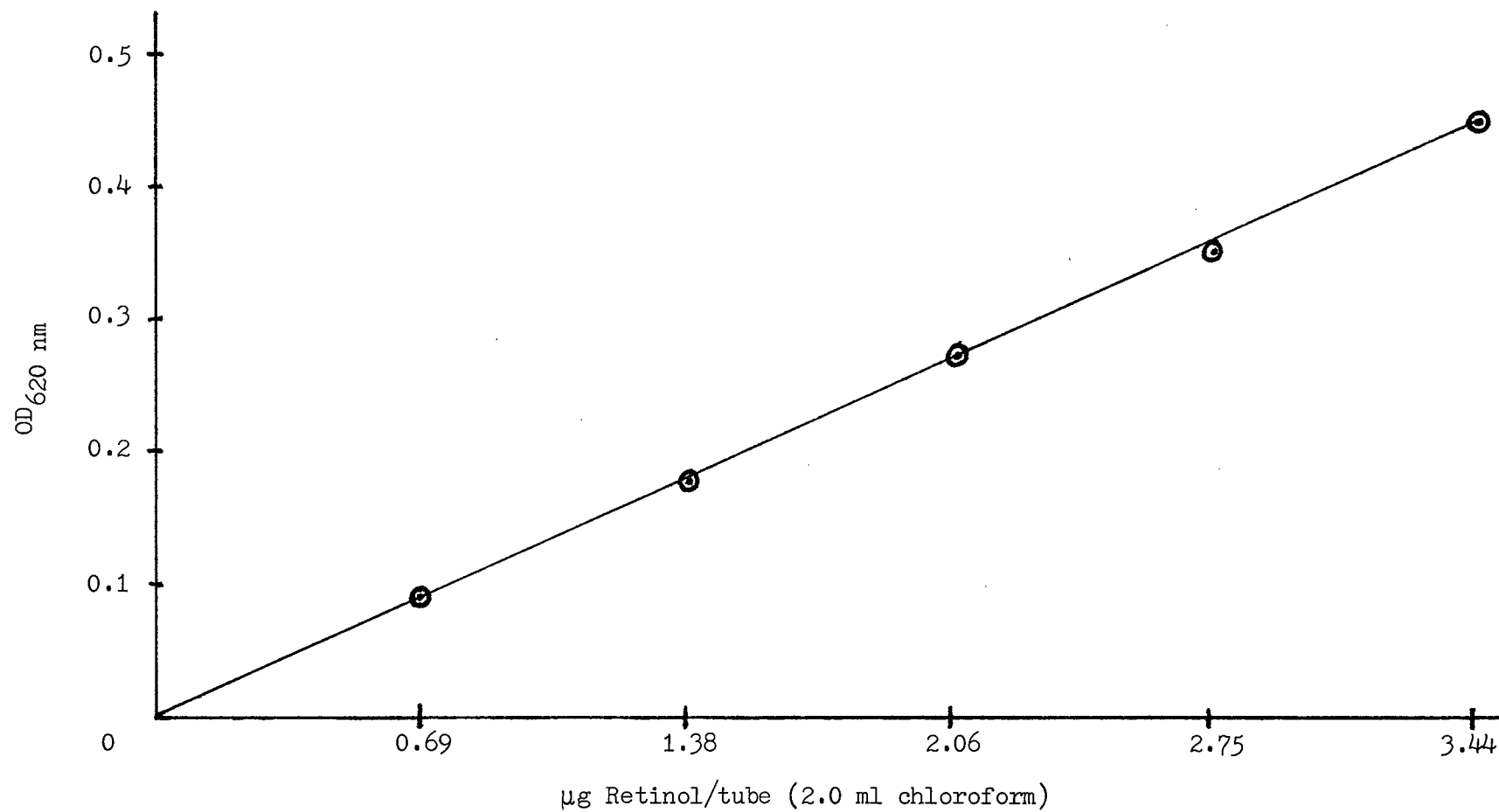
#### 6. Plasma Vitamin E

Plasma vitamin E levels were determined according to the method described by Fabianek et al. (1968), which is a modification of the classic



FIGURE 3

STANDARD CURVE FOR PLASMA VITAMIN A



Emmerie-Engel technique. The analysis is based on a reduction of ferric ion to the ferrous form by tocopherols, with the resultant formation of a pink complex of ferrous ions with 4,7-diphenyl-10,10-phenanthroline. The use of phosphoric acid prevents the photochemical reduction of ferric chloride and also reduces interference of carotene to a minimum. The complex was measured with a Beckman DU-2 spectrophotometer at 536 nm.

Standard curves for mg dl- $\alpha$ -tocopherol/100 ml plasma were established using dl- $\alpha$ -tocopherol (Hoffmann-La Roche Inc., Nutley, N.J., U.S.A.). The average slope of the curve at 536 nm was found to be 2.54 (Fig. 4).

The plasma tocopherol concentration was then calculated from the standard curve (Fig. 4) and expressed as mg tocopherol per 100 ml of plasma.

#### 7. Plasma Cholesterol

Plasma cholesterol was assayed by an enzymatic color procedure described by Roschlau et al. (1974). A 0.02 ml aliquot of plasma was mixed with 5 ml of cholesterol reagent mixture<sup>1</sup> (1.7 M methanol; 0.57 M ammonium phosphate buffer, pH 7; 0.02 M acetylacetone; 0.1% hydroxypolyethoxydodecane; catalase > 670 U/ml; cholesterol-esterase > 26 mU/ml). The contents of the test tubes were mixed well using a Vortex mixer and 0.02 ml of cholesterol oxidase (4 U/ml) was added. The samples were incubated at 37°C for 60 minutes and the optical density was read at 410 nm against a sample blank on a Beckman DU-2 spectrophotometer.

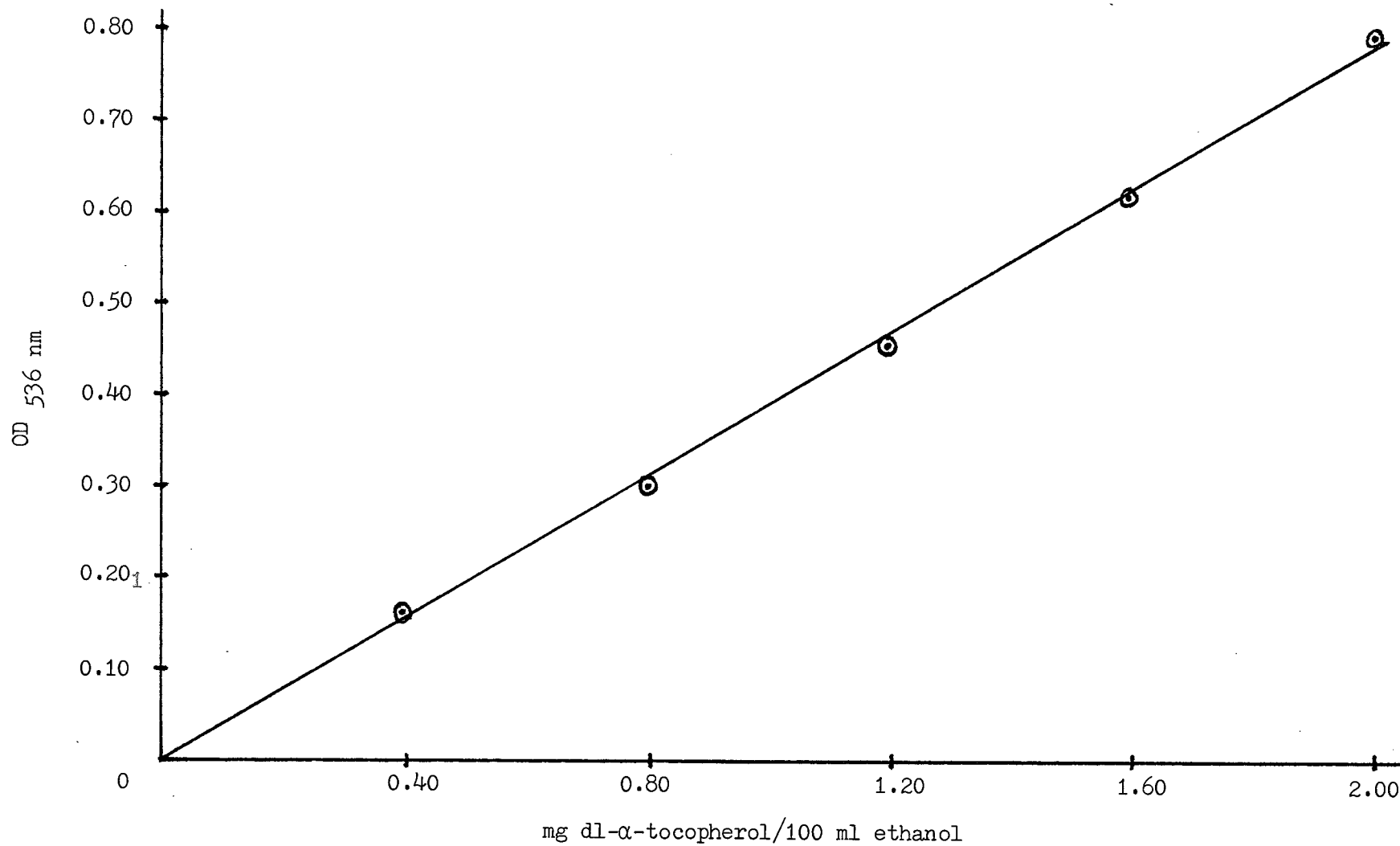
Standard curves for mg cholesterol/100 ml plasma were determined using pure cholesterol (Preciset Cholesterol<sup>1</sup>). The average slope of the

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<sup>1</sup> Boehringer Mannheim GmbH, Mannheim, W. Germany

FIGURE 4

STANDARD CURVE FOR PLASMA VITAMIN E



curve at 410 nm was found to be 818.1 (illustrated in Figure 5).

The plasma cholesterol levels were calculated as shown below.

$$\text{mg cholesterol/100 ml plasma} = \text{OD}_{410 \text{ nm}} \times 818.1$$

#### 8. Plasma Total Lipids

Total lipids in plasma were measured by the method of Amenta (1970). Lipids were extracted from the plasma into a chloroform-methanol solution 1.5:1 (V/V) and non-lipid impurities and methanol were removed by a wash with an aqueous  $\text{CaCl}_2$  solution (0.5%). An aliquot of the lipid-containing chloroform phase was evaporated and the total lipid measured by reacting with an acid dichromate reagent (0.5%). The amount of dichromate reduced was determined by the change in absorption measured at 430 nm on a Beckman DU=2 spectrophotometer which was directly proportional to the lipid present. The standard for total lipids was lecithin (0.1%) and palmitic acid (0.15%), dissolved in chloroform.

Total lipids in plasma were then determined according to the formula shown below.

$$\text{mg total lipids/100 ml plasma} = \frac{A_x}{A_s} \times Z$$

where

$$A_x = \text{OD}_{430 \text{ nm}} \text{ method blank} - \text{OD}_{430 \text{ nm}} \text{ sample}$$

$$A_s = \text{OD}_{430 \text{ nm}} \text{ reagent blank} - \text{OD}_{430 \text{ nm}} \text{ standard}$$

$$Z = \text{concentration of the standard} \times \text{dilution factor}$$

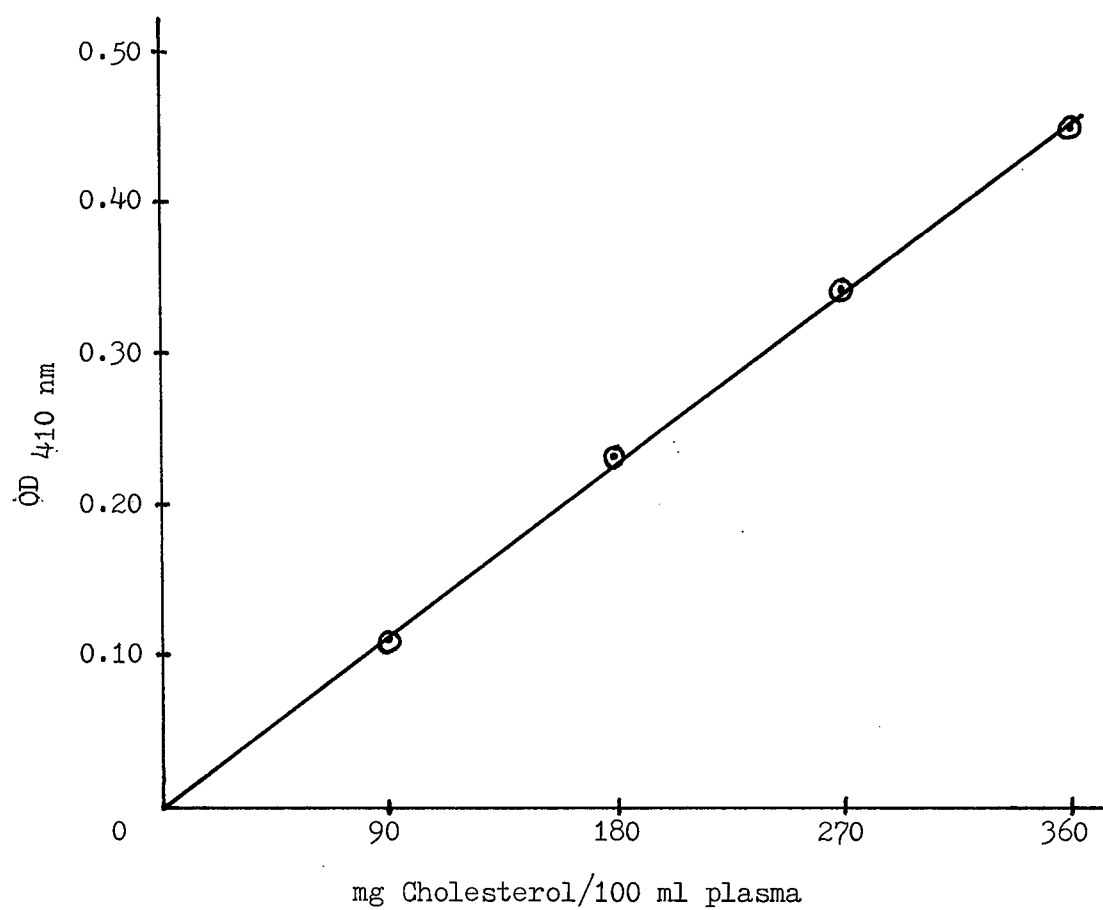
#### 9. Plasma Alkaline Phosphatase

Plasma alkaline phosphatase was assayed by a procedure described by Henry et al. (1974).

A 0.1 ml aliquot of plasma was mixed with 1 ml of 0.02 M phenol

FIGURE 5

## STANDARD CURVE FOR PLASMA CHOLESTEROL



phosphate. The hydrolysis product, phenol, was condensed with 4-aminoantipyrine and then oxidized with alkaline ferricyanide to give a red complex which was measured at 500 nm on a Beckman DU-2 spectrophotometer.

One unit of alkaline phosphatase activity was defined as the amount of enzyme in 100 ml of plasma which liberated 1 mg phenol in 15 minutes at 37°C.

The amount of alkaline phosphatase in the plasma was then calculated as follows.

$$\text{units alkaline phosphatase/100 ml plasma} = \frac{A_x - A_c}{A_s} \times Z$$

where

$A_x$  = absorbance of unknown at 500 nm

$A_c$  = absorbance of control at 500 nm

$A_s$  = absorbance of standard at 500 nm

$Z$  = concentration of the standard x dilution factor

#### 10. Liver Lipid Extraction

The concentrations of vitamin A, cholesterol and total lipids in ~~liver of rats~~ liver of rats were measured in the chloroform-extract of liver, prepared by a modification of the methods of Folch et al. (1957) and Amenta (1970). The lipid extraction procedure was carried out as follows.

One half g of liver was minced and then homogenized in 1 ml distilled water, first with a Sorvall micro-homogenizer attached to a Sorvall omni-mixer and then with a Potter-Elvehjem glass and teflon plunger type of homogenizer. One half milliliter of crude homogenate was extracted with 3 ml of chloroform-methanol 1.5:1 (V/V) in a glass stoppered centrifuge tube by agitating vigorously for 3 minutes with a

Vortex mixer. The tubes were then centrifuged at 1,200 x G for 5 minutes. The upper chloroform phase was pipetted off and retained. The supernatant phase was extracted with 3 ml of chloroform-methanol 1.5:1 (V/V) as before and recentrifuged. The liquid phase was combined with the chloroform phase from the first extraction. The mixture was then washed with 3 ml of aqueous  $\text{CaCl}_2$  solution (67.5 mM) by shaking vigorously for 3 minutes and then centrifuged at 1,200 x G. Aliquots of the lipid-containing chloroform phase were then ready for the vitamin A, cholesterol and total lipid analyses.

#### 11. Liver Vitamin A

The level of vitamin A in liver was determined according to the method of Neeld and Pearson (1973). An aliquot of the lipid-containing chloroform phase was diluted 1:3 with chloroform, from which 0.2 ml was used for the vitamin A analysis. The blue chromophore produced by the interaction of trifluoroacetic acid and vitamin A in chloroform extract was measured at 620 nm on a Beckman DU-2 spectrophotometer.

Standard curves for retinol equivalents per tube were established using all trans retinyl acetate (Hoffmann-La Roche Inc.). The average slope of the curve at 620 nm was found to be 7.19. An illustration of this calibration curve is shown in Figure 6.

In the preliminary laboratory work, known amounts of all trans retinyl acetate were added to liver before the lipid extraction procedure. Analysis was carried out according to the method discussed above and the per cent recovery was calculated. It was found that recovery of 103 per cent was attained.

The vitamin A concentration in liver was then calculated from the

standard curve (Fig. 6) and expressed as  $\mu\text{g}$  per g of liver.

## 12. Liver Vitamin E

The level of  $\alpha$ -tocopherol in liver was determined according to the thin-layer chromatography (TLC) method of Bieri (1969).

Two-dimensional analysis was carried out on precoated silica gel G TLC plates (Redi/Plate, Fisher Scientific) using benzene-ethanol (99:1) and hexane-ethanol (9:1) mixtures as solvents. After the solvent had evaporated from the second dimension run the chromatograms were sprayed with a 0.0025% solution of sodium fluorescein in methanol. This aided in visualization and identification of the  $\alpha$ -tocopherol spot.

Following elution, a colorimetric determination of the  $\alpha$ -tocopherol in the ethanol eluate was carried out. The method essentially consisted of extracting the ethanol eluate with xylene, followed by the addition of 0.4% 4,7-diphenyl-10,10-phenanthroline, 0.6% ferric chloride and 85% orthophosphoric acid.

Standard curves for  $\mu\text{g}$   $\alpha$ -tocopherol per tube were established using dl- $\alpha$ -tocopherol (Hoffmann-La Roche Inc.). The average slope of the curve at 536 nm was found to be 10.2 (illustrated in Fig. 7).

In the preliminary laboratory work, known amounts of dl- $\alpha$ -tocopherol were added to liver from vitamin E-free treated rats prior to saponification. Analysis was carried out and the per cent recovery was determined. It was found that up to 84.6 per cent recovery could be obtained. Consequently a correction factor of 1.18 was employed to compensate for this loss.

The  $\alpha$ -tocopherol concentration in liver was then calculated from the standard curve (Fig. 7) and expressed as  $\mu\text{g}$  per g of liver and also as  $\mu\text{g}$  per whole liver.



FIGURE 6

STANDARD CURVE FOR LIVER VITAMIN A

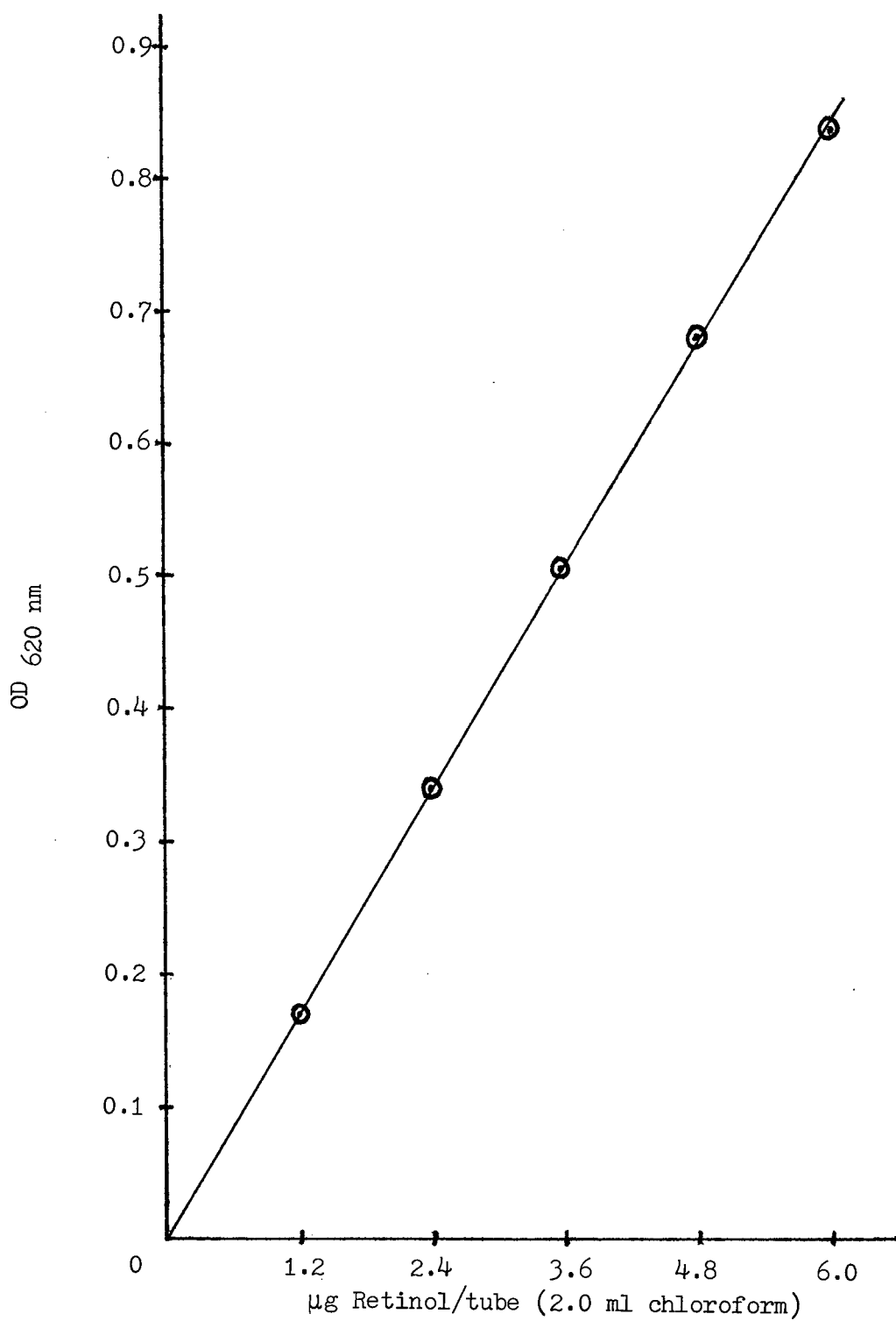
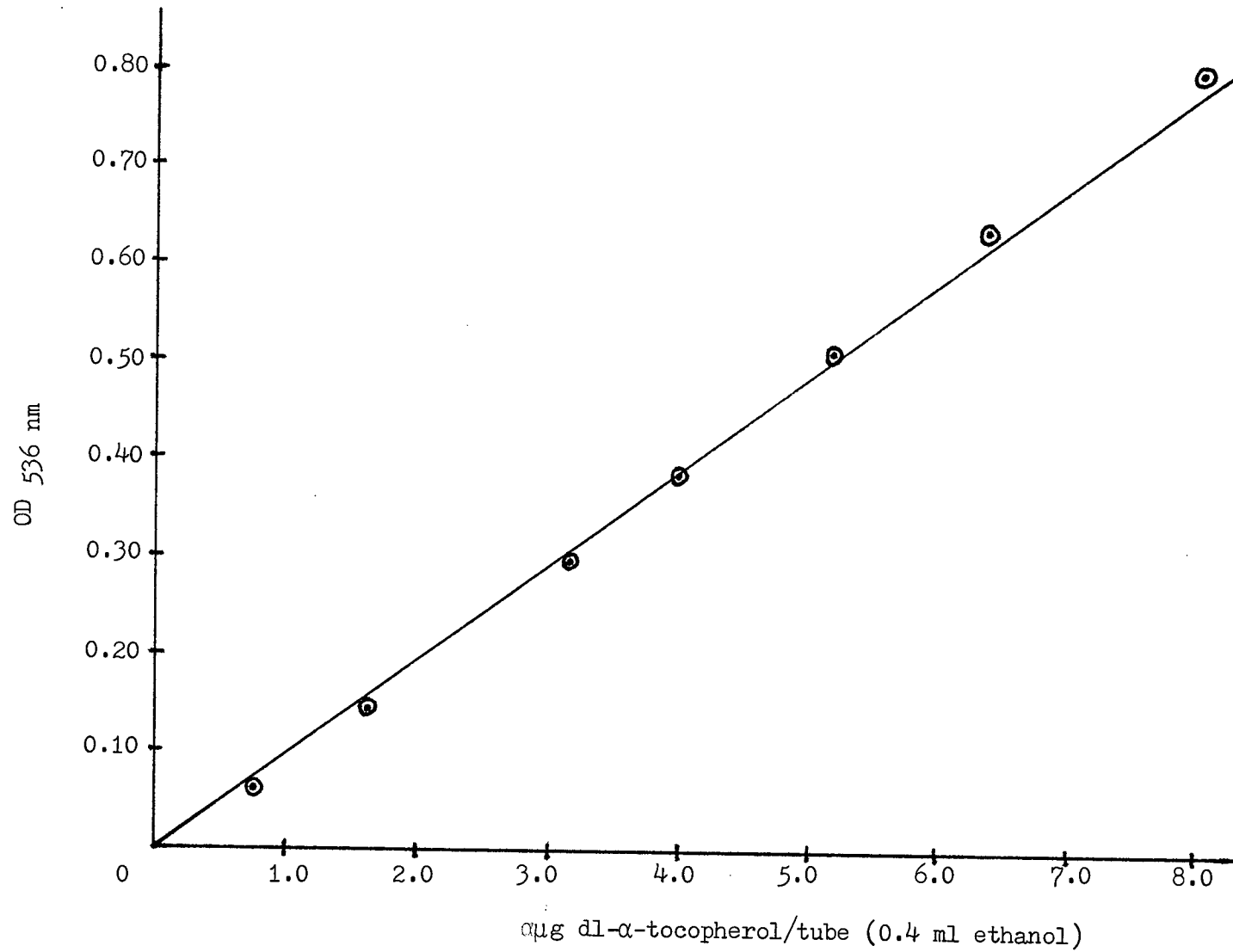


FIGURE 7

STANDARD CURVE FOR LIVER VITAMIN E



### 13. Liver Total Lipids

Total lipids in liver were determined by the method described by Amenta (1970). One half milliliter of the lipid-containing chloroform phase was mixed with 1.5 ml chloroform, from which 0.4 ml was evaporated and the total lipid measured by reacting with an acid dichromate reagent. The amount of dichromate reduced was determined by the change in absorption when measured at 430 nm on a Beckman DU-2 spectrophotometer and was directly proportional to the amount of lipid present.

In the preliminary laboratory work, known amounts of lipid were added to liver before the lipid extraction procedure. Analysis was carried out according to the method discussed above and the per cent recovery was calculated. It was found that the recovery of 101 per cent was attainable.

Total lipids in liver were calculated by the same formula used for determining plasma total lipids (See Section 8.) and were expressed as follows.

$$\text{mg total lipid/g liver}$$

### 14. Liver Cholesterol

Total cholesterol in liver was determined by the enzymatic color test of Roschlau et al. (1974). A 1.5 ml aliquot of the lipid-containing chloroform phase was evaporated to dryness by flushing with nitrogen in a test tube placed in a heating block set at 50°C. The cholesterol-residue was dissolved in 5 ml of cholesterol reagent mixture (See Section 7, Plasma Cholesterol for a description of the reagent mixture) by sonic treatment at 0°C. The contents of the test tube were mixed well using a Vortex mixer and 0.02 ml of cholesterol-oxidase (4 U/ml) was added. The samples were incubated at 37°C for 60 minutes and the optical density was

read at 410 nm against a sample blank on a Beckman DU-2 spectrophotometer.

Calibration curves of  $\mu\text{g}$  cholesterol per tube were established using pure cholesterol (Preciset Cholesterol, Boehringer Mannheim GmbH) as standard. The calibration factor of the curve at 410 nm was found to be 368.1 (illustrated in Figure 8).

In the preliminary laboratory work, known amounts of cholesterol were added to liver before the lipid extraction procedure. Analysis was carried out according to the method discussed above and per cent recovery was calculated. It was found that recovery of 103 per cent was attained.

The cholesterol content in the liver was calculated from the standard curve (Fig. 8) and expressed as mg per g of liver.

#### 15. Femoral Ash

The femoral bone was ashed by a modification of the procedure of Zipken et al. (1959). The femur was first weighed, then defatted with a mixture of chloroform-methanol 2:1 (V:V) for 24 hours. The defatted bone was dried in an isothermal oven at  $105^{\circ}\text{C}$  for 24 hours, weighed and then ashed in a furnace at  $650^{\circ}\text{C}$  for 18 hours. The bone ash was weighed and then dissolved in 4 ml of 3 N HCl.

The per cent ash of the femur was calculated as shown below.

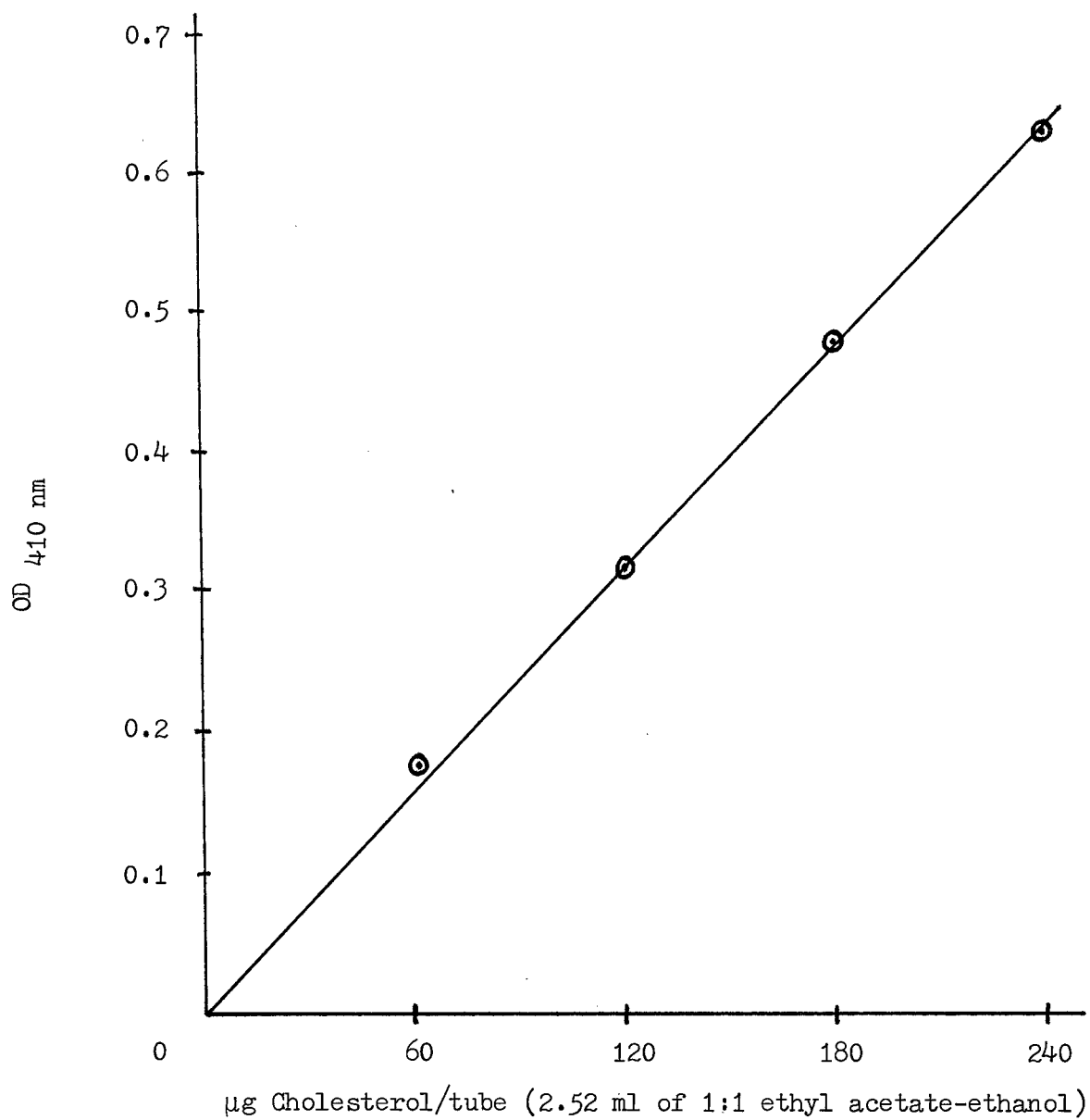
$$\text{per cent ash} = \frac{\text{weight of ash (g)}}{\text{weight of defatted dry bone (g)}} \times 100$$

#### 16. Femoral Calcium

The calcium in bone was determined by atomic absorption spectrophotometry using a method described by Willis (1960). The whole dried femoral bone dissolved in 3 N HCl was diluted with lanthanum chloride solution ( $5\% \text{La}^{3+}$ ) so that the calcium concentration lay between 5 and 20 mg/l.

FIGURE 8

STANDARD CURVE FOR LIVER CHOLESTEROL



Analysis was carried out using a Unicam SP90 Atomic Absorption Spectrophotometer.

Calibration curves for mg per cent calcium were determined using AnalaR calcium carbonate (Canadina Lab. Supplies Ltd.) in AnalaR hydrochloric acid. The calibration factor of the curve at 422.7 mμ was found to be 6.67. An illustration of this calibration curve is shown in Figure 9.

The total calcium in the femur was calculated from the calibration curve (Fig. 9) and expressed as g calcium per femur. The per cent calcium in the dry and defatted femur was calculated as shown below.

$$\begin{array}{l} \text{per cent} \\ \text{calcium} \\ \text{in femur} \end{array} = \frac{\text{weight of calcium in femur (g)}}{\text{weight of defatted dry femur (g)}} \times 100$$

#### 17. Femoral Inorganic Phosphate

Inorganic phosphate in the femur was determined by a modified method of Fiske and Subbarow (1970).

The inorganic phosphate analysis of the diluted bone-HCl solution involved photometric determination of the molybdenum blue formed by reduction of the molybdenum diphosphate, using aminonaphtholsulfonic acid as the reducing agent.

Calibration curves for μg phosphate per tube were determined using a phosphorus standard (5 μg/ml). The calibration factor of the curve at 660 nm was found to be 35.8 (illustrated in Fig. 10).

The total inorganic phosphate in the femur was calculated from the calibration curve (Fig. 10) and expressed as g inorganic phosphate per femur. The per cent phosphate in the dry defatted femur was calculated as shown below.

FIGURE 9

STANDARD CURVE FOR CALCIUM

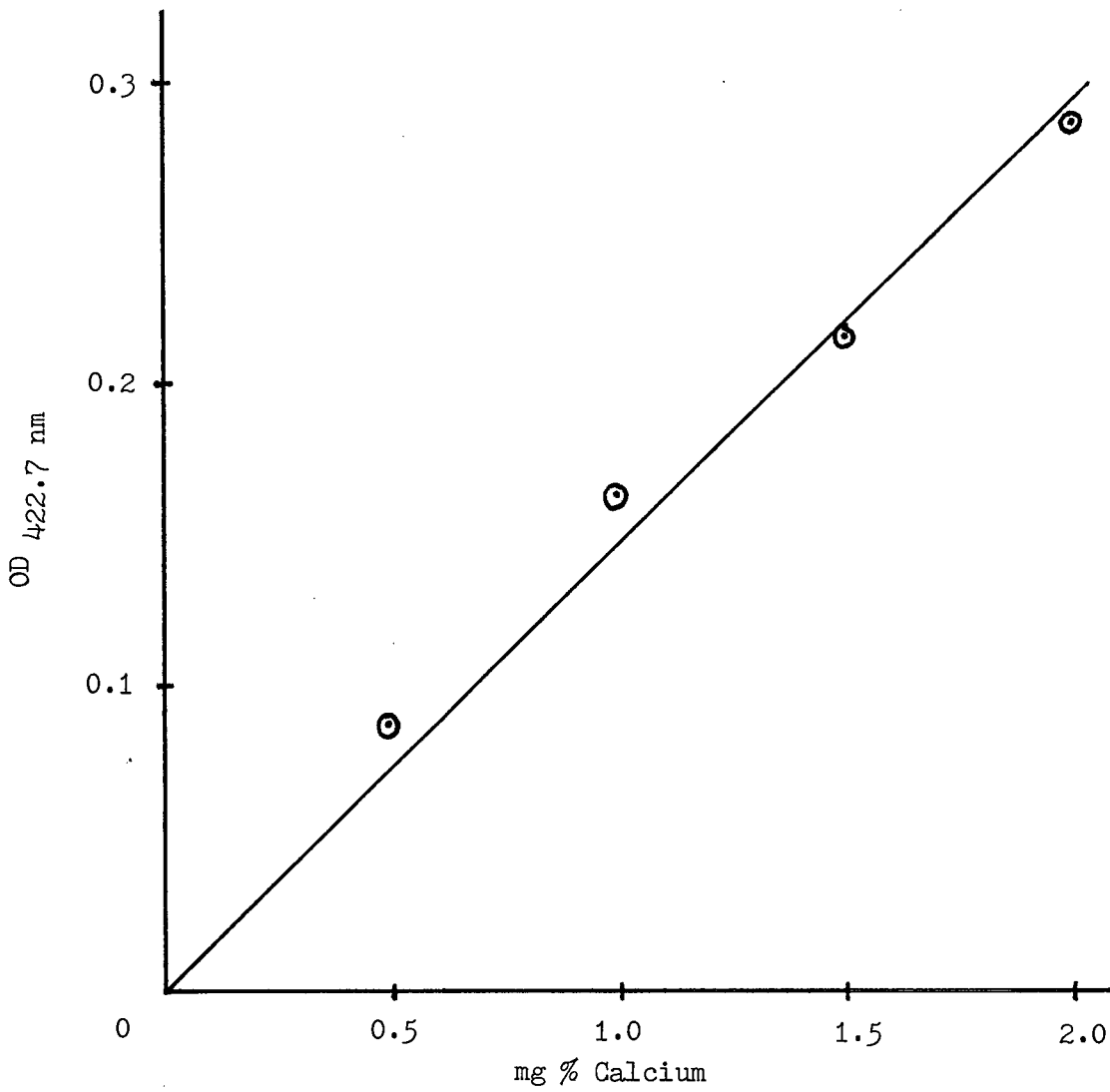
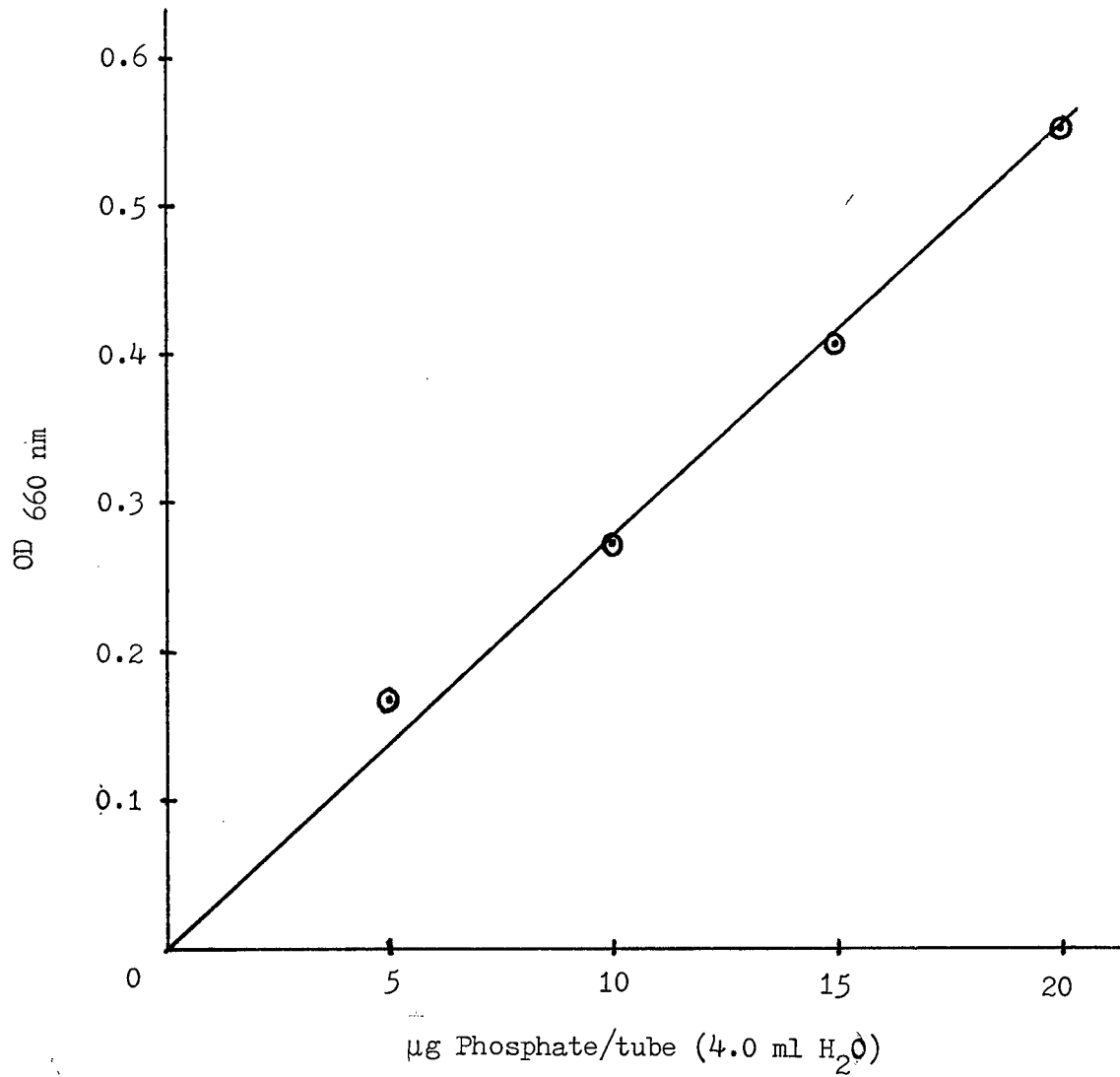


FIGURE 10

## STANDARD CURVE FOR PHOSPHATE





$$\begin{array}{l} \text{per cent} \\ \text{phosphate} \\ \text{in femur} \end{array} = \frac{\text{weight of phosphate in femur (g)}}{\text{weight of defatted dry femur (g)}} \times 100$$

#### F. Statistical Analysis of the Data

The raw data were analyzed statistically by computer at the Computing Centre of the University of British Columbia. The SPSS computer program package (Kita, 1976) was employed to draw up a program for the desired analyses.

Experimental data were tested by applying one-way analysis of variance. The homogeneity of variances was tested by Cochran's C test. A log transformation was used for data with heterogeneous variance. Statistical comparisons were made using regression analysis and Duncan's new multiple-range test for data containing equal number of samples among groups or by Scheffe's range test for data containing unequal number of samples on a probability level of at least 95 per cent for all measurements.

## CHAPTER IV

## RESULTS

A. Body and Organ Weights

Results of the effect of different levels of dietary vitamin E on growth in rats treated for 8 and 16 months are presented in Tables 3 and 4 respectively. As can be seen in Table 3, high levels of vitamin E had a significant depressing effect ( $P < 0.05$ ) on growth of rats treated for 8 months. Vitamin E deficiency resulted in a greater growth depression than excess dietary vitamin E supplementation. The results in Table 4 also show that growth rate was significantly reduced in rats treated with high levels of dietary vitamin E for 16 months.

The organ weights (expressed in mg/100 g body weight) of rats treated with different levels of vitamin E for 8 and 16 months are also shown in Tables 3 and 4. Treatment for 8 months at all levels of vitamin E supplementation had no significant effect on liver, uterus, spleen or kidney weight. However, increasing the dietary level of vitamin E was found to significantly increase ( $P < 0.05$ ) the relative heart weights in rats treated for 8 months. The linear relationship between relative heart weights (Y) and log dietary vitamin E (X) was  $Y = 232.316 + 11.347X$ , the correlation coefficient (R) being 0.465 ( $P < 0.05$ ). Results for the relative organ weights of the vitamin E deficient rats show that all organs, except uterus, were significantly larger ( $P < 0.01$ ) than those receiving vitamin E treatment.

Table 4 shows that treatment for 16 months at all levels of vitamin E supplementation had no significant effect on the relative weights of liver, uterus or kidney. However, a significant increase

TABLE 3  
Body and Organ Weights of Rats on Different  
Levels of Dietary Vitamin E For 8 Months<sup>1</sup>

Dietary vitamin E	Body weight	Liver	Uterus	Heart	Spleen	Kidney
IU/kg diet	g	mg/100g body weight				
0	166 <sup>a</sup> (13)	5,215 <sup>a</sup> (292)	183 (27)	416 <sup>a</sup> (24)	313 <sup>a</sup> (26)	1,159 <sup>a</sup> (32)
25	366 <sup>bd</sup> (31)	2,790 <sup>b</sup> (123)	189 (20)	248 <sup>b2</sup> (16)	160 <sup>b</sup> (4)	536 <sup>b</sup> (42)
250	332 <sup>cd</sup> (12)	2,470 <sup>b</sup> (70)	204 (25)	269 <sup>b2</sup> (12)	161 <sup>b</sup> (8)	560 <sup>b</sup> (30)
2,500	356 <sup>bd</sup> (16)	2,469 <sup>b</sup> (204)	198 (13)	251 <sup>b2</sup> (10)	164 <sup>b</sup> (5)	496 <sup>b</sup> (32)
10,000	313 <sup>cd</sup> (8)	2,540 <sup>b</sup> (102)	176 (19)	282 <sup>b2</sup> (13)	159 <sup>b</sup> (14)	576 <sup>b</sup> (29)
25,000	301 <sup>c</sup> (16)	2,451 <sup>b</sup> (157)	197 (16)	289 <sup>b2</sup> (10)	167 <sup>b</sup> (6)	547 <sup>b</sup> (19)

<sup>1</sup>Values are means of four rats with their SEM given in parentheses. Values within each column not sharing a common superscript letter are significantly different ( $P < 0.05$ ) using Duncan's new multiple-range test.

<sup>2</sup>Linear response significant ( $P < 0.05$ ) against dietary vitamin E using regression analysis. The functional relationship between relative heart weights (Y) and log dietary vitamin E (X) was  $Y = 232.316 + 11.347 X$ , the correlation coefficient (R) being 0.465 ( $P < 0.05$ ).

TABLE 4

Body and Organ Weights of Rats on Different  
Levels of Dietary Vitamin E For 16 Months<sup>1</sup>

Dietary vitamin E	Number rats	Body weight	Liver	Uterus	Heart <sup>2</sup>	Spleen <sup>3</sup>	Kidney
IU/kg diet		g					
0	—	—	—	—	—	—	—
25	4	408 <sup>a</sup> (12)	1,957 (109)	165 (25)	268 (8)	167 (14)	599 <sup>ab</sup> (25)
250	9	457 <sup>a</sup> (20)	2,702 (166)	217 (20)	229 (9)	168 (6)	501 <sup>a</sup> (20)
2,500	6	414 <sup>a</sup> (17)	3,088 (175)	214 (14)	263 (7)	184 (11)	586 <sup>ab</sup> (41)
10,000	7	398 <sup>ab</sup> (21)	3,065 (107)	202 (19)	268 (6)	218 (14)	583 <sup>ab</sup> (21)
25,000	5	358 <sup>b</sup> (10)	2,873 (135)	248 (22)	294 (6)	240 (34)	627 <sup>b</sup> (21)

<sup>1</sup>Values are means with their SEM given in parentheses. Values within each column not sharing a common superscript letter are significantly different ( $P < 0.05$ ) using Scheffe's range test.

<sup>2</sup>Quadratic response significant ( $P < 0.01$ ) against dietary vitamin E. The functional relationship between relative heart weight (Y) and log dietary vitamin E (X) was  $Y = 355.603 - 85.825X + 16.219X^2$ , the multiple correlation coefficient (R) being 0.649 ( $P < 0.01$ ).

<sup>3</sup>Linear response significant ( $P < 0.008$ ) against dietary vitamin E using regression analysis. The functional relationship between relative spleen weight (Y) and log dietary vitamin E (X) was  $Y = 2.127 + 0.051X$ , where  $R = 0.580$  ( $P < 0.008$ ).

( $P < 0.01$ ) in relative heart weight was shown in rats fed high levels of vitamin E for 16 months. The functional relationship between relative heart weights (Y) and log dietary vitamin E (X) was  $Y = 355.603 - 85.825X + 16.219X^2$ , the multiple correlation coefficient (R) being 0.649 ( $P < 0.01$ ). The relative spleen weights were also significantly increased ( $P < 0.008$ ) in rats fed high levels of vitamin E for 16 months. The linear relationship between relative spleen weight (Y) and log dietary vitamin E (X) was  $Y = 2.127 + 0.051X$ , where  $R = 0.580$  ( $P < 0.008$ ).

#### B. Hematological Parameters

The influence of treatment with different levels of dietary vitamin E for 9, 12 and 16 months on hemoglobin and hematocrit values, erythrocyte hemolysis and prothrombin time are presented in Tables 5, 6, 7 and 8 respectively.

There was no significant difference in hemoglobin levels (Table 5) when the rats were fed different dietary levels of vitamin E for 9, 12 and 16 months.

The hematocrit values of rats treated with different levels of vitamin E are shown in Table 6. There were no significant differences in the hematocrit values of rats treated for 9 or 12 months. However, the hematocrit values were significantly increased ( $P < 0.04$ ) by treatment with excess vitamin E for 16 months. The functional relationship between hematocrit value (Y) and log dietary vitamin E (X) was  $Y = 41.018 + 1.242X$ , where  $R = 0.482$  ( $P < 0.04$ ).

Results of the effect of different levels of dietary vitamin E on the spontaneous hemolysis of the erythrocytes in a buffered isotonic saline solution in rats treated for 9, 12 and 16 months are presented in

TABLE 5  
Hemoglobin Values<sup>1</sup> of Rats Fed Different  
Levels of Dietary Vitamin E

Dietary vitamin E	Feeding period (months)		
	9	12	16
IU/kg diet	(4)	(3)	(4)
0	15.9± 0.4	—	—
25	15.3± 0.1	15.2± 0.3	14.8± 0.5
250	15.9± 0.1	15.5± 0.7	15.5± 0.4
2,500	15.6± 0.2	15.0± 0.4	14.8± 0.3
10,000	15.6± 0.1	14.5± 1.2	15.1± 0.5
25,000	14.6± 0.3	15.7± 0.4	12.4± 2.2

<sup>1</sup> Each value represents mean ± SEM for the number of rats given in parentheses above each column.

TABLE 6  
Hematocrit Values<sup>1</sup> of Rats Fed Different  
Levels of Dietary Vitamin E

Dietary vitamin E	Feeding period (months)		
	9	12	16 <sup>2</sup>
IU/kg diet	Hematocrit (%)		
	(4)	(3)	(4)
0	45.6± 0.9	—	—
25	45.5± 0.5	44.7± 1.2	42.6± 0.8
250	45.1± 0.9	44.3± 0.6	45.4± 0.7
2,500	45.2± 0.7	42.9± 1.1	42.6± 0.8
10,000	44.2± 0.6	43.4± 3.1	46.6± 1.0
25,000	44.1± 1.1	46.2± 0.7	47.2± 2.0

<sup>1</sup> Each value represents mean ± SEM for the number of rats given in parentheses above each column.

<sup>2</sup> Linear response significant ( $P < 0.04$ ) against dietary vitamin E using regression analysis. The functional relationship between hematocrit value (Y) and log dietary vitamin E was  $Y = 41.018 + 1.242 X$ , where  $R = 0.482$  ( $P < 0.04$ ).

TABLE 7  
Erythrocyte Hemolysis<sup>1</sup> of Rats Fed  
Different Levels of Dietary Vitamin E

Dietary vitamin E	Feeding period (months)		
	9	12	16
IU/kg diet	Hemolysis (%)		
	(4)	(3)	(4)
0	86.8± 2.4 <sup>a</sup>	—	—
25	2.4± 0.5 <sup>b</sup>	2.2± 0.3	3.7± 0.6 <sup>a</sup>
250	1.6± 0.2 <sup>b</sup>	2.3± 0.4	2.6± 0.2 <sup>ab</sup>
2,500	1.8± 0.4 <sup>b</sup>	2.7± 0.7	3.4± 0.6 <sup>ab</sup>
10,000	2.0± 0.1 <sup>b</sup>	2.0± 0.1	2.1± 0.4 <sup>b</sup>
25,000	1.7± 0.2 <sup>b</sup>	1.4± 0.3	3.7± 0.3 <sup>a</sup>

<sup>1</sup> Each value is the mean ± SEM for the number of rats given in parentheses above each column. Values within each column not sharing a common superscript letter are significantly different (P<0.05) using Duncan's new multiple-range test.



TABLE 8

Prothrombin Times of Rats Fed Different  
Levels of Dietary Vitamin E

Dietary vitamin E	Feeding period (months)		
	9	12 <sup>2</sup>	16 <sup>3</sup>
IU/kg diet	Prothrombin Time (sec)		
	(4)	(3)	(4)
0	13.1± 0.2	—	—
25	14.9± 0.8	14.6± 0.1	13.0± 0.4
250	12.9± 0.4	15.6± 0.5	14.5± 0.2
2,500	13.8± 0.5	14.3± 0.7	13.6± 0.6
10,000	13.9± 0.5	11.3± 0.3	12.8± 0.5
25,000	14.2± 0.6	11.3± 0.4	12.7± 0.3

<sup>1</sup> Each value is the mean ± SEM for the number of rats give in parentheses above each column.

<sup>2</sup> Quadratic response significant ( $P < 0.0001$ ) against dietary vitamin E. The functional relationship between prothrombin time (Y) and log dietary vitamin E (X) was  $Y = 10.409 + 4.439X - 0.991X^2$ , where  $R = 0.894$  ( $P < 0.0001$ ).

<sup>3</sup> Quadratic response significant ( $P < 0.02$ ) against dietary vitamin E using regression analysis. The functional relationship between prothrombin time (Y) and log dietary vitamin E (X) was  $Y = 9.819 + 3.208X - 0.597X^2$ , where  $R = 0.609$  ( $P < 0.02$ ).

Table 7. Vitamin E supplementation did not significantly affect the stability of the erythrocyte at any period of treatment. Vitamin E deficiency for 9 months significantly increased the fragility of the erythrocyte membrane.

The prothrombin time values of rats treated with different levels of vitamin E are shown in Table 8. Treatment for 9 months at all levels of vitamin E supplementation had no significant effect on prothrombin time. In rats treated with high dietary levels of vitamin E for 12 and 16 months the prothrombin time was significantly shorter. In rats treated for 12 months the functional relationship between prothrombin time (Y) and log dietary vitamin E (X) was  $Y = 10.409 + 4.439X - 0.991X^2$ , where  $R = 0.894$  ( $P < 0.0001$ ). In rats treated for 16 months the functional relationship between prothrombin time (Y) and log dietary vitamin E (X) was  $Y = 9.819 + 3.208X - 0.597X^2$ , where  $R = 0.609$  ( $P < 0.02$ ).

#### C. Femoral Parameters

The influence of excess vitamin E administration for 8 and 16 months on ash content and calcium and phosphate concentration of bone are shown in Tables 9 and 10. Included in Table 10 is the plasma alkaline phosphatase activity which was measured at 16 months as an additional parameter of bone calcification.

Treatment with different levels of vitamin E for 8 months did not significantly affect the ash content of the femur. (Table 9). However, after 16 months of high dietary vitamin E supplementation (Table 10) the ash content of bone decreased significantly ( $P < 0.0005$ ). The functional relationship between the percentage ash content of bones (Y) and log dietary vitamin E (X) was  $Y = 68.970 - 1.207X$ , where  $R = 0.703$  ( $P < 0.0005$ ).

TABLE 9

Femoral Parameters of Rats Fed Different Levels  
of Dietary Vitamin E for 8 Months

Dietary vitamin E	Feeding period : 8 months		
	Ash	Calcium	Phosphate
IU/kg diet	%	%	%
0	65.3± 0.7	23.4± 0.3 <sup>ab</sup>	12.6± 0.4 <sup>a</sup>
25	65.5± 0.2	22.9± 0.3 <sup>ab</sup>	12.0± 0.3 <sup>ab</sup>
250	66.2± 0.3	23.6± 0.3 <sup>ab</sup>	11.8± 0.1 <sup>ab</sup>
2,500	65.7± 0.7	22.8± 0.5 <sup>a</sup>	11.3± 0.2 <sup>b</sup>
10,000	66.0± 0.9	23.9± 0.4 <sup>b</sup>	11.9± 0.2 <sup>ab</sup>
25,000	66.3± 0.5	23.0± 0.2 <sup>ab</sup>	11.5± 0.1 <sup>b</sup>

<sup>1</sup>Each value is the mean ± SEM for 4 rats. Values within each column not sharing a common superscript letter are significantly different (P<0.05) using Duncan's new multiple-range test.

TABLE 10

Femoral Parameters of Rats Fed Different Levels  
of Dietary Vitamin E For 16 Months

Dietary		Feeding period : 16 months		
vitamin E	Ash <sup>2</sup>	Calcium	Phosphate	Plasma alkaline phosphatase <sup>3,4</sup>
IU/kg diet	%	%	%	units/100 ml
0	—	—	—	—
25	67.4± 0.5	23.1± 0.5	11.9± 0.1 <sup>ab</sup>	17.9± 1.1
250	65.9± 0.4	21.9± 0.8	11.6± 0.6 <sup>ab</sup>	14.1± 1.8
2,500	65.0± 0.6	23.6± 1.1	12.4± 0.6 <sup>b</sup>	15.7± 0.1
10,000	64.2± 1.1	22.5± 0.5	10.9± 0.3 <sup>a</sup>	22.1± 4.6
25,000	63.6± 1.0	21.0± 0.3	11.4± 0.3 <sup>ab</sup>	24.6± 4.3

<sup>1</sup>Each value is the mean ± SEM for four rats. Values within each column not sharing a common superscript letter are significantly different ( $P < 0.05$ ) using Duncan's new multiple-range test.

<sup>2</sup>Linear response significant ( $P < 0.0005$ ) against dietary vitamin E. The functional relationship between the percentage ash content of bones (Y) and log dietary vitamin E (X) was  $Y = 68.970 - 1.207X$ , where  $R = 0.703$  ( $P < 0.0005$ ).

<sup>3</sup>Quadratic response significant ( $P < 0.04$ ) against dietary vitamin E using regression analysis. The functional relationship between plasma alkaline phosphatase activity (Y) and log dietary vitamin E (X) was  $Y = 13.789 + 0.468X^2$ , where  $R = 0.463$  ( $P < 0.04$ ).

<sup>4</sup>One unit of alkaline phosphatase activity was defined as the amount of enzyme in 100 ml of plasma which liberated 1 mg phenol in 15 minutes at 37°C.

Femoral calcium content was not significantly affected by excess vitamin E supplementation for either 8 or 16 months.

There were some slight, but significant differences ( $P < 0.05$ ) in femoral phosphate concentration in rats treated with different levels of vitamin E for 8 and 16 months. Regression analysis though was unable to show any significant relationship between the phosphate concentration and the dietary level of vitamin E supplemented.

As can be seen in Table 10, excess vitamin E supplementation, from 250 to 25,000 IU/kg diet increased plasma alkaline phosphatase activity after 16 months treatment. The functional relationship between plasma alkaline phosphatase activity (Y) and log dietary vitamin E (X) was  $Y = 13.789 + 0.469X^2$ , where  $R = 0.463$  ( $P < 0.04$ ).

#### D. Urinary Creatine and Creatinine

Data from the analysis of urinary creatine and creatinine of rats treated with different levels of vitamin E for 11 months are presented in Table 11. Vitamin E supplementation at all levels did not influence urinary levels of either creatine or creatinine. Vitamin E deficiency significantly increased ( $P < 0.01$ ) the urinary creatine excretion, while the urinary creatinine excretion decreased significantly ( $P < 0.01$ ).

#### E. Fat Soluble Vitamins

##### 1. b Liver and Plasma Vitamin E

The influence of high levels of dietary vitamin E on liver storage of  $\alpha$ -tocopherol after 8 and 16 months of treatment is shown in Table 12. The results are reported as both, the  $\alpha$ -tocopherol concentration of the liver ( $\mu\text{g/g}$  liver) and the total  $\alpha$ -tocopherol content of the liver ( $\mu\text{g/whole liver}$ ).

TABLE 11

Urinary creatine and creatinine of rats on different  
levels of dietary vitamin E for 11 months<sup>1</sup>

Dietary vitamin E	Creatine	Creatinine	Creatine/Creatinine
IU/kg diet	mg/kg/24 hr	mg/kg/24 hr	ratio
0	54.07±19.90 <sup>a</sup>	14.50± 2.31 <sup>a</sup>	5.04± 2.86 <sup>a</sup>
25	3.14± 0.62 <sup>b</sup>	27.50± 1.95 <sup>b</sup>	0.11± 0.03 <sup>b</sup>
250	1.44± 0.20 <sup>b</sup>	27.12± 1.32 <sup>b</sup>	0.05± 0.01 <sup>b</sup>
2,500	1.92± 0.39 <sup>b</sup>	27.83± 1.28 <sup>b</sup>	0.07± 0.02 <sup>b</sup>
10,000	3.20± 0.46 <sup>b</sup>	25.72± 0.60 <sup>b</sup>	0.12± 0.02 <sup>b</sup>
25,000	1.94± 0.38 <sup>b</sup>	27.11± 1.49 <sup>b</sup>	0.07± 0.02 <sup>b</sup>

<sup>1</sup>Each value is the mean ± SEM for 4 rats. Values within each column not sharing a common superscript letter are significantly different (P<0.01) using Duncan's new multiple-range test.

As can be seen in Table 12, increasing the level of dietary vitamin E up to 10,000 IU/kg diet for 8 and 16 months significantly increased ( $P < 0.0001$ ) the vitamin E concentration of the liver. Additional vitamin E supplementation, above 10,000 IU/kg diet, did not significantly increase the liver vitamin E concentration any further. Analysis of the data revealed a linear relationship between the dietary levels of vitamin E and the vitamin E concentration in liver when both were expressed as logarithms (Fig. 11). The logarithmic relationship between liver vitamin E and dietary vitamin E was 94% linear for rats treated for 8 months and 98% linear for rats treated for 16 months. The functional relationship between log liver vitamin E (Y) and log dietary vitamin E (X) in rats treated for 8 months was  $Y = -0.106 + 1.252X - 0.107X^2$ , where  $R = 0.980$  ( $P < 0.0001$ ); and in rats treated for 16 months was  $Y = 0.762 + 0.652X$ , where  $R = 0.988$  ( $P < 0.0001$ ).

Shown in Figure 12 are the concentrations of vitamin E in plasma of rats fed different dietary levels of vitamin E for 8 and 16 months. In rats treated for 8 months plasma levels of vitamin E were significantly increased ( $P < 0.005$ ) with increasing levels of dietary vitamin E intake. The functional relationship between log plasma vitamin E (Y) and log dietary vitamin E (X) in rats treated for 8 months was  $Y = -1.419 + 0.949X - 0.132X^2$ , where  $R = 0.84$  ( $P < 0.005$ ). The plasma vitamin E levels were over 2-fold higher in rats fed various levels of vitamin E for 16 months than those fed for 8 months. The functional relationship between log plasma vitamin E (Y) and log dietary vitamin E (X) in rats treated for 16 months was  $Y = -0.622 + 0.595X - 0.064X^2$ , where  $R = 0.96$  ( $P < 0.00001$ ).

## 2. Liver and Plasma Vitamin A

The effect of different levels of dietary vitamin E on the

TABLE 12

The Concentrations of  $\alpha$ -Tocopherol in Livers of Rats Fed Different Levels of Dietary Vitamin E For 8 and 16 Months

Dietary Vitamin E	Liver $\alpha$ -tocopherol <sup>1</sup>			
	8 months		16 months	
IU/kg diet	$\mu\text{g/g liver}^2$	$\mu\text{g/whole liver}$	$\mu\text{g/g liver}^3$	$\mu\text{g/whole liver}$
0	0.5 $\pm$ 0.1	4.6 $\pm$ 1.1	—	—
25	27.1 $\pm$ 1.8	277.5 $\pm$ 33.5	43.7 $\pm$ 1.4	577.6 $\pm$ 29.7
250	204.9 $\pm$ 15.5	1,691.9 $\pm$ 182.0	214.2 $\pm$ 17.1	2,535.7 $\pm$ 296.4
2,500	703.0 $\pm$ 88.7	6,104.4 $\pm$ 882.3	1,172.6 $\pm$ 181.7	14,323.8 $\pm$ 2,150.2
10,000	1,952.3 $\pm$ 203.1	15,403.5 $\pm$ 1,456.7	2,822.6 $\pm$ 283.1	30,994.7 $\pm$ 5,826.5
25,000	2,214.7 $\pm$ 587.8	17,137.3 $\pm$ 6,572.0	3,411.1 $\pm$ 513.1	37,236.6 $\pm$ 6,268.2

<sup>1</sup> Each value represents mean  $\pm$  SEM for four rats.

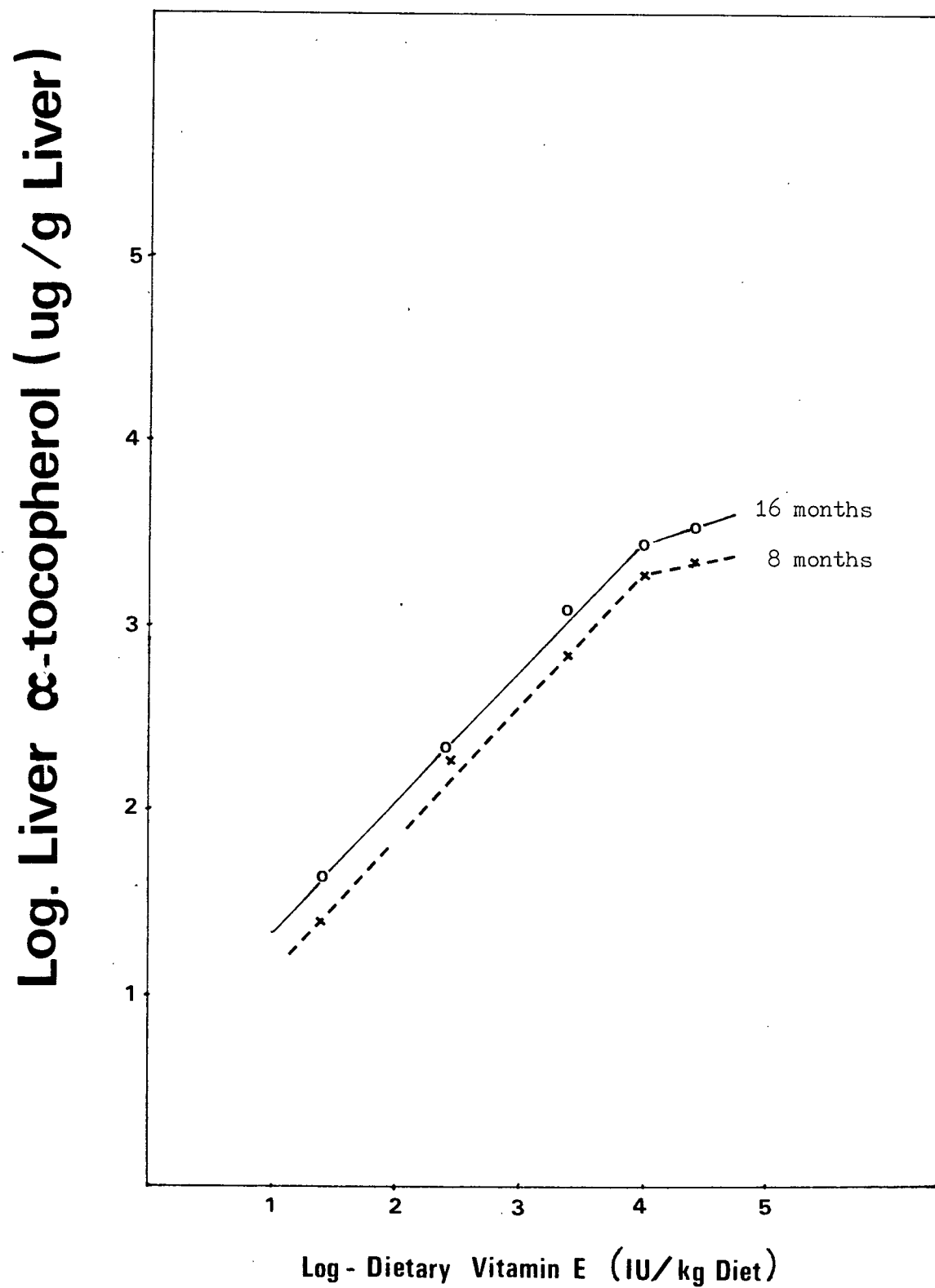
<sup>2</sup> Quadratic response significant ( $P < 0.0001$ ) against dietary vitamin E in supplemented rats.

<sup>3</sup> Linear response significant ( $P < 0.0001$ ) against dietary vitamin E.



FIGURE 11

Plot of the logarithm of liver  $\alpha$ -tocopherol concentration  
versus the logarithm of dietary vitamin E, in rats  
treated for 8 and 16 months.



## FIGURE 12

Plasma  $\alpha$ -tocopherol concentration of rats fed different dietary levels of vitamin E for 8 months (open bars) and 16 months (closed bars).

Each point represents mean  $\pm$  SEM of four rats.

In rats treated for 8 months plasma tocopherol in group A is significantly lower than other groups ( $P < 0.01$ ) using Duncan's multiple-range test.

In rats treated for 8 months, quadratic response is significant ( $P < 0.005$ ) against dietary vitamin E.

In rats treated for 16 months, quadratic response is significant ( $P < 0.0001$ ) against dietary vitamin E.

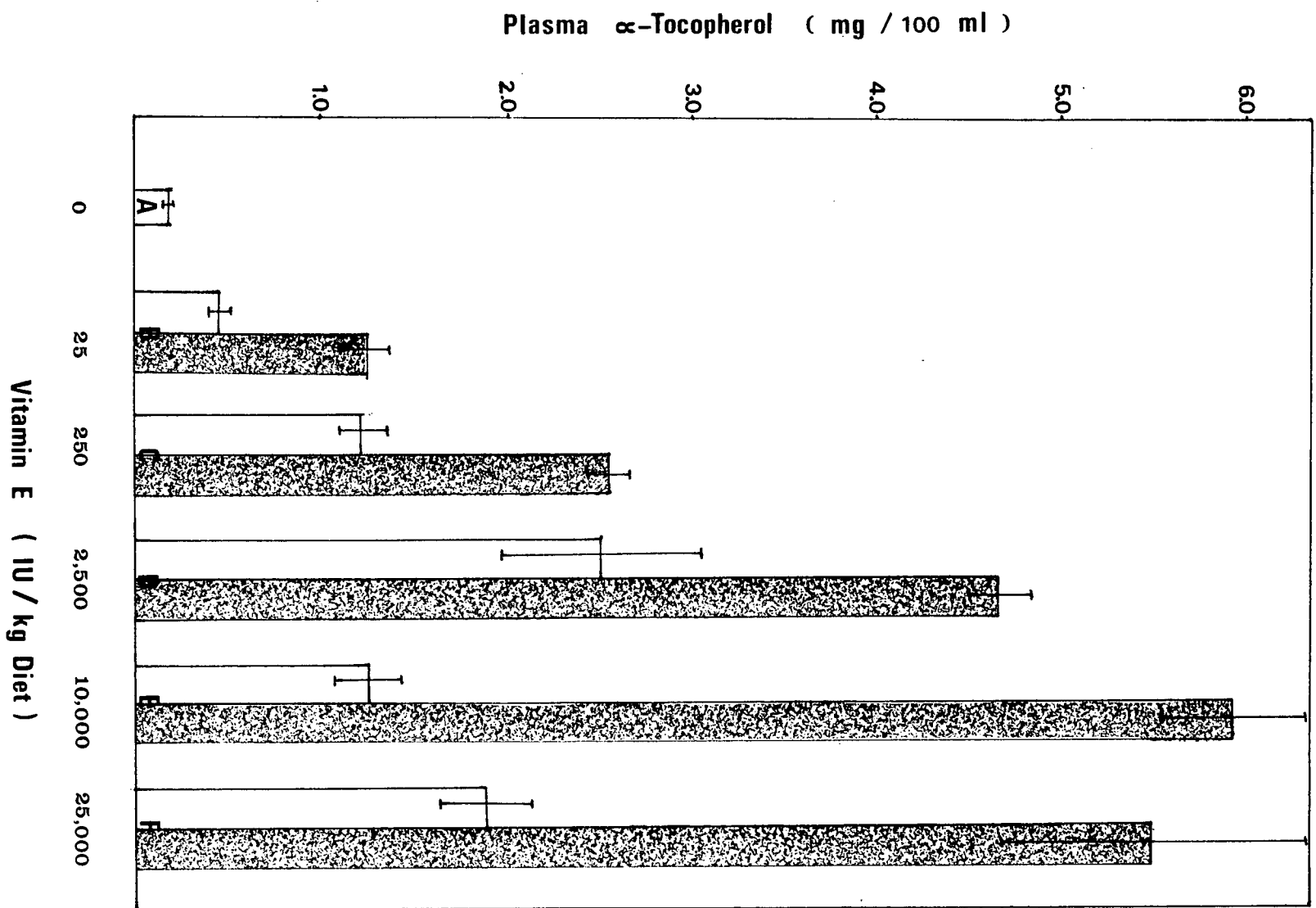


FIGURE 13

Liver vitamin A concentration of rats fed different dietary levels of vitamin E for 8 months (open bars) and 16 months (closed bars).

Each point represents mean  $\pm$  SEM of four rats.

In rats treated for 8 months group A is significantly lower than all other groups ( $P < 0.01$ ).

In rats treated for 16 months group C is significantly higher than groups D, E and F ( $P < 0.05$ ).

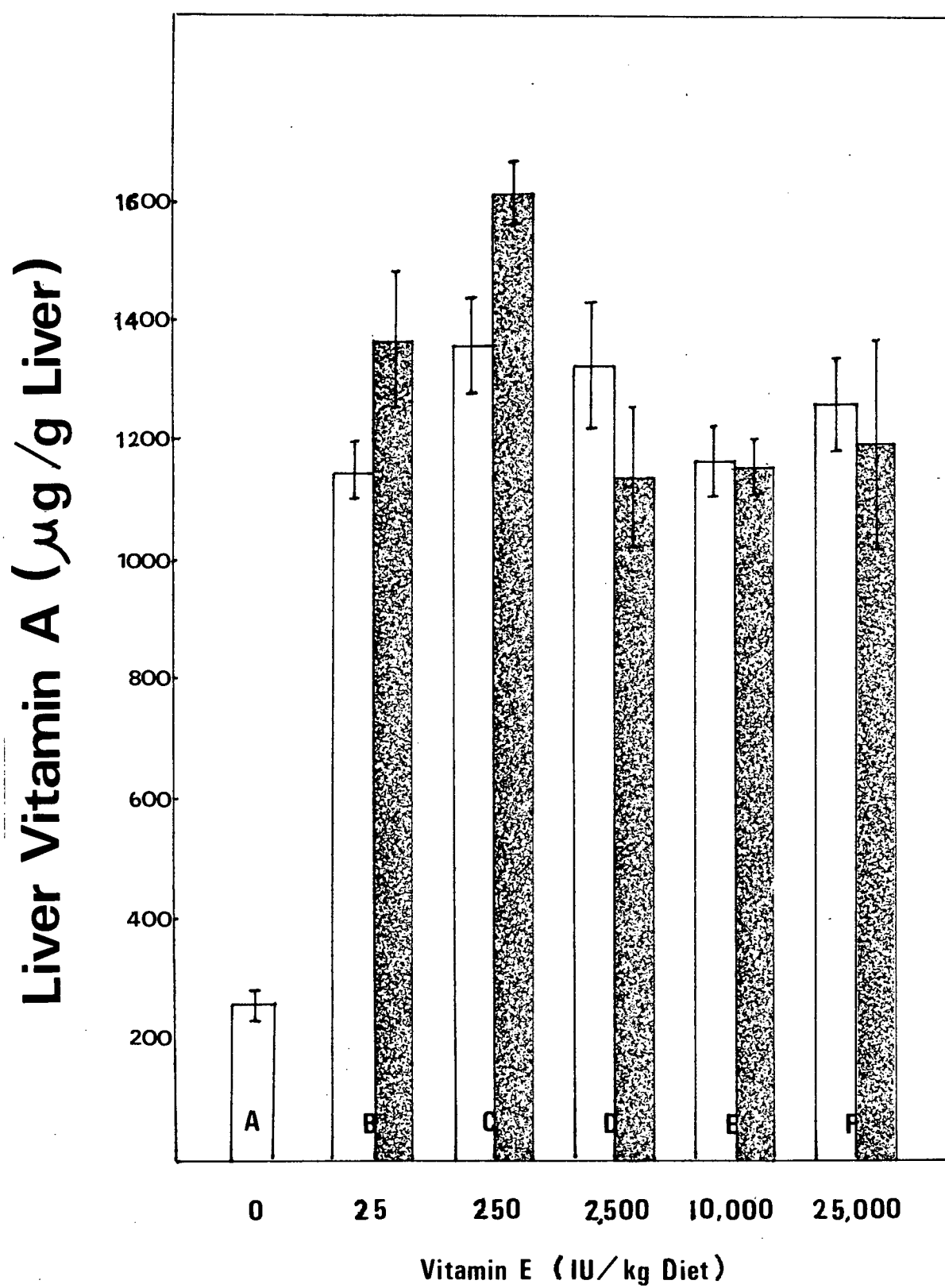
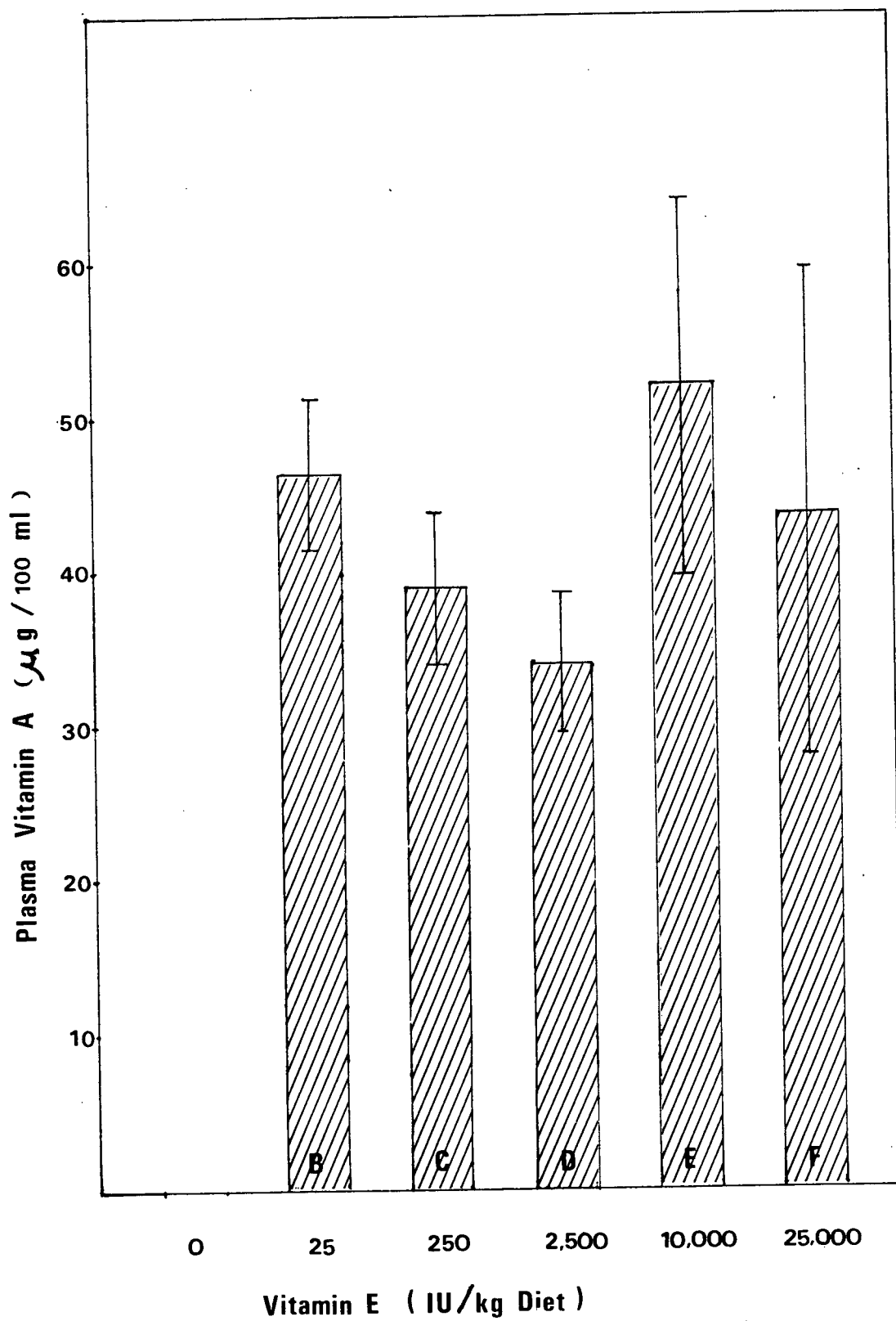


FIGURE 14

Plasma vitamin A concentration of rats fed different dietary levels of vitamin E for 16 months.

Each point represents mean  $\pm$  SEM of four rats.





concentration of vitamin A in liver is shown in Figure 13. Supplementation with high levels of vitamin E for 8 and 16 months had no significant influence on liver vitamin A storage. However, after 8 months, vitamin E deficiency was found to significantly decrease ( $P < 0.01$ ) the liver vitamin A storage.

The influence of high levels of vitamin E on plasma vitamin A levels was examined only after 16 months treatment (Fig. 14). Long-term treatment with excess vitamin E had no significant effect on plasma vitamin A levels.

#### F. Lipids

##### 1. Liver Total Lipids and Cholesterol"

The influence of high levels of vitamin E on liver concentrations of total lipids and cholesterol are presented in Figures 15 and 16 respectively.

The liver total lipids were significantly increased ( $P < 0.0001$ ) after 8 months treatment with high levels of dietary vitamin E (Fig. 15). The liver total lipids were increased from 4.5% of the liver weight in rats fed 25 IU vitamin E/kg diet to 14.0% in rats fed 25,000 IU vitamin E/kg diet. The linear relationship between log liver lipids (Y) and log dietary vitamin E (X) was  $Y = 1.497 + 0.144X$ , where  $R = 0.86$  ( $P < 0.0001$ ). With a longer experimental period - 16 months, it was interesting to observe that there was no significant difference in liver total lipids at different levels of vitamin E supplementation. All groups treated for 16 months, except those fed 25 IU vitamin E/kg diet, had lower lipid levels than those groups treated for 8 months at comparable levels of vitamin E supplementation.

FIGURE 15

Total lipids in liver of rats fed different dietary levels of vitamin E for 8 months (open bars) and 16 months (closed bars).

Each point represents mean  $\pm$  SEM of four rats.

Group A is significantly lower than other groups ( $P < 0.01$ ) using Duncan's new multiple-range test in rats fed for 8 months.

In rats supplemented for 8 months, linear response is significant ( $P < 0.0001$ ) against dietary vitamin E.

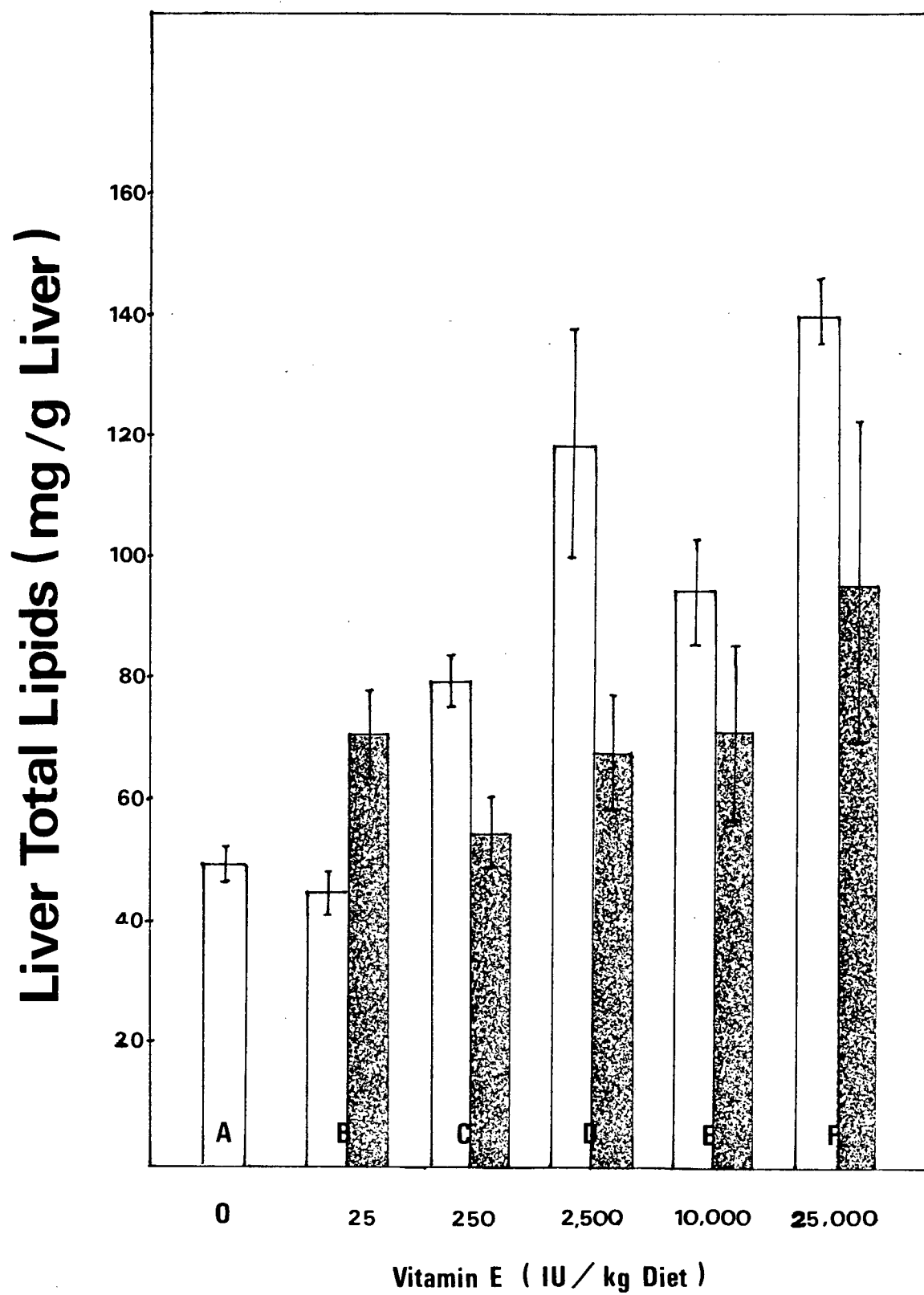
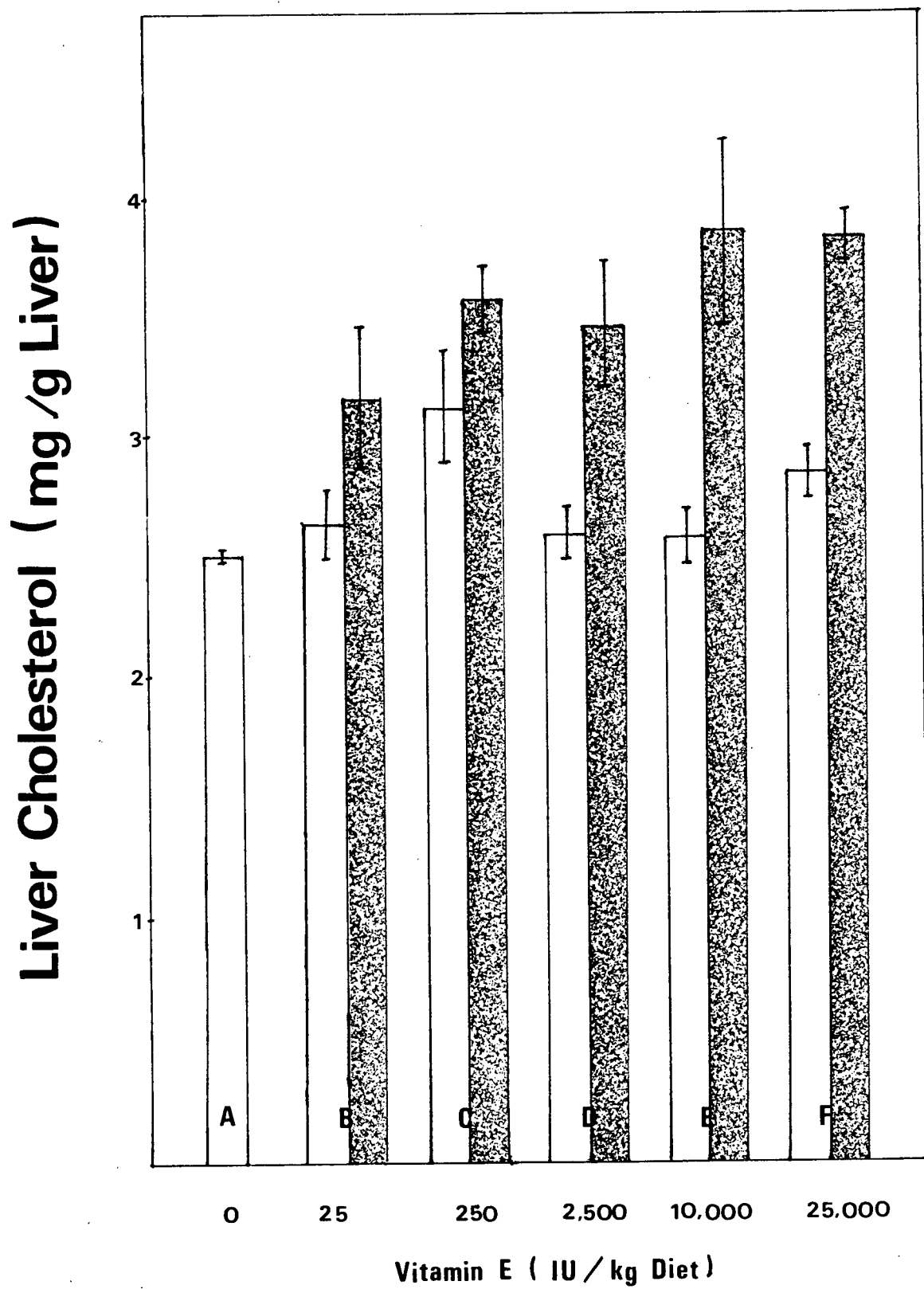


FIGURE 16

Liver cholesterol concentration of rats fed different dietary levels of vitamin E for 8 months (open bars) and 16 months (closed bars).

Each point represents mean  $\pm$  SEM of four rats.

Group C is significantly higher than other groups ( $P < 0.05$ ) except group F rats fed for 8 months.



As shown in Figure 16, liver cholesterol was unaffected by high levels of vitamin E after 8 and 16 months of treatment. In contrast to the results of liver total lipids, rats treated for 16 months had generally higher liver cholesterol levels than those treated for 8 months.

## 2. Plasma Total Lipids and Cholesterol

Results of the effect of different levels of dietary vitamin E on plasma total lipids and cholesterol are shown in Figure 17 and 18 respectively.

Treatment with high levels of dietary vitamin E for 8 months had no significant effect on plasma total lipids. However, the plasma total lipids of rats fed 25 or 250 IU vitamin E/kg diet for 8 months were higher than in rats fed more than 2,500 IU/kg diet. This was also observed in the rats treated for 16 months. Regression analysis revealed that increasing the dietary level of vitamin E significantly decreased ( $P < 0.024$ ) the plasma total lipids in rats treated for 16 months. The linear relationship between log plasma lipids (Y) and log dietary vitamin E (X) in rats treated for 16 months was  $Y = 3.0306 - 0.00814X$ , where  $R = 0.41$  ( $P < 0.024$ ).

Vitamin E supplementation for 8 months had no significant effect on plasma cholesterol (Fig. 18). The plasma cholesterol levels of vitamin E deficient rats was significantly lower ( $P < 0.05$ ) than those supplemented with 2,500 IU vitamin E/kg diet for 8 months.

Treatment with excess levels of dietary vitamin E, 2,500 IU/kg diet or higher for 16 months significantly lowered ( $P < 0.05$ ) the plasma cholesterol level (Fig. 18).

FIGURE 17

Total lipids in plasma of rats fed different dietary levels of vitamin E for 8 months (open bars) and 16 months (closed bars).

Each point represents mean  $\pm$  SEM of number of rats shown in parentheses.

In rats treated for 16 months, linear response is significant ( $P < 0.024$ ) against dietary vitamin E.

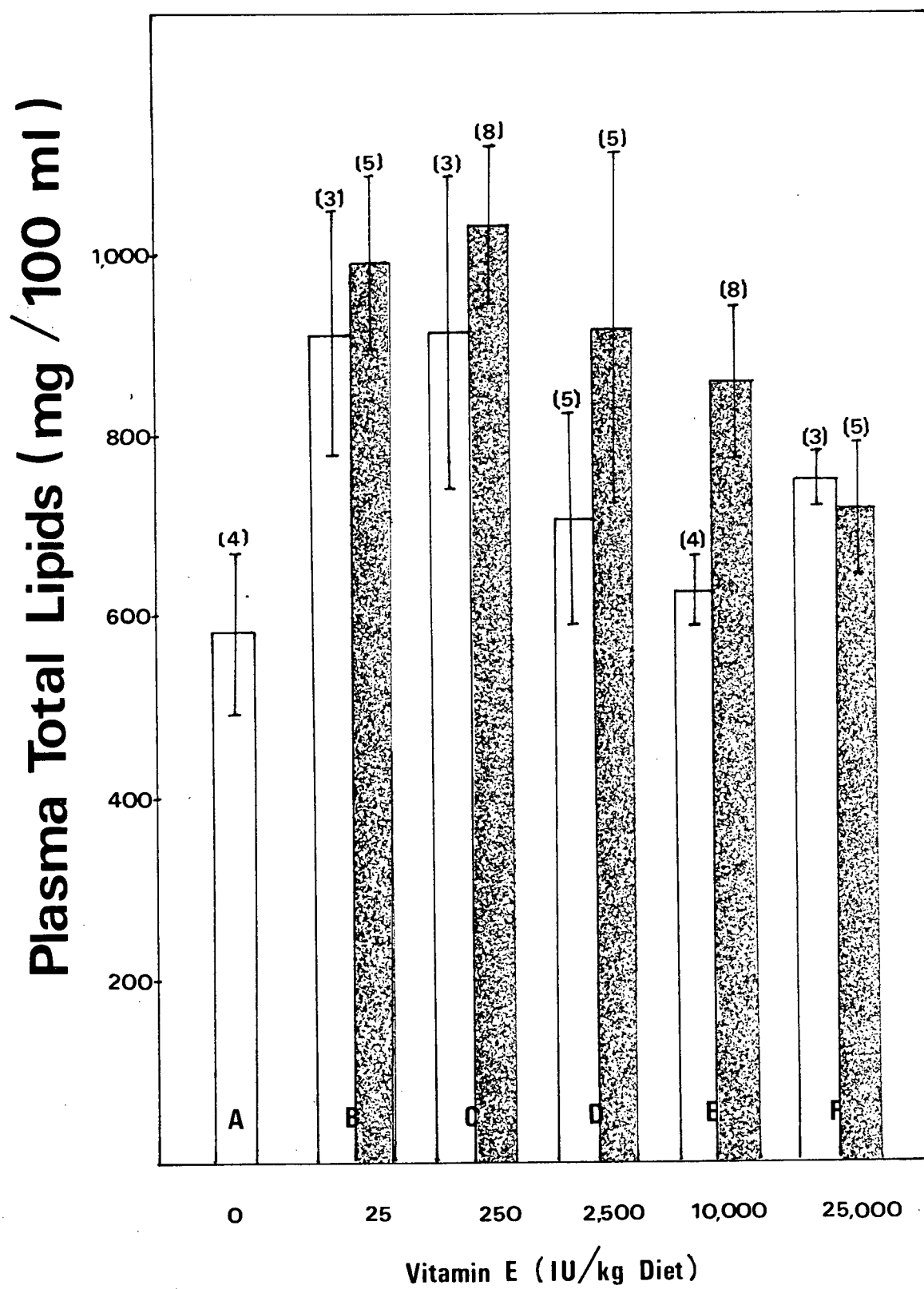




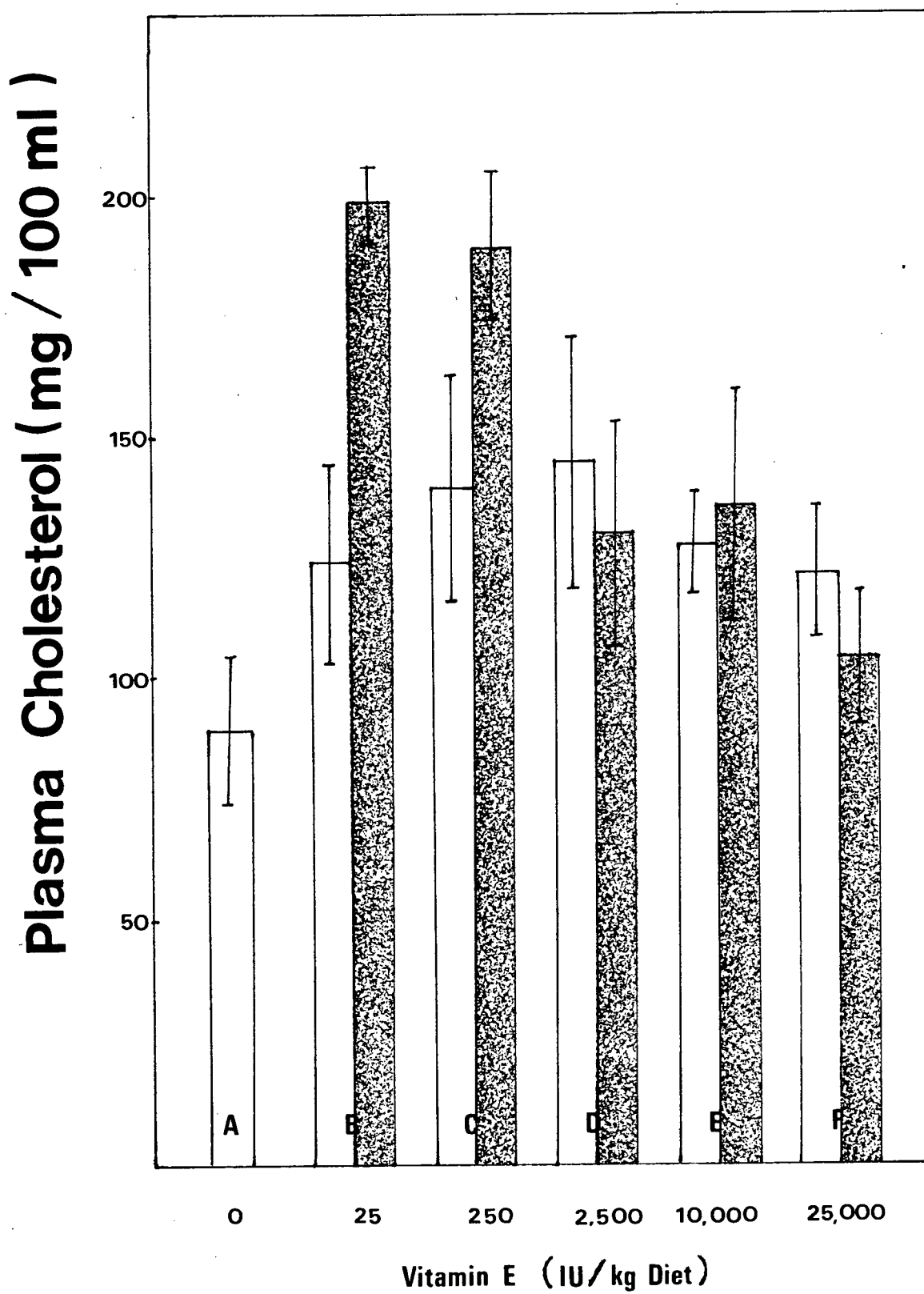
FIGURE 18

Cholesterol concentration in plasma of rats fed different dietary levels of vitamin E for 8 months (open bars) and 16 months (closed bars).

Each point represents mean  $\pm$  SEM of four rats.

In rats treated for 8 months, group D is significantly higher ( $P < 0.05$ ) than group A.

In rats treated for 16 months, groups B and C are significantly higher ( $P < 0.05$ ) than groups D, E and F.



## CHAPTER V

## DISCUSSION

A. Body and Organ Weights

The body weights of rats treated with high levels of vitamin E (10,000 and 25,000 IU/kg diet) for 8 months were significantly depressed ( $P < 0.05$ ). Body weights were also significantly reduced in rats fed 25,000 IU vitamin E/kg diet for 16 months compared to those receiving 25 to 2,500 IU/kg diet. From these results it appears that excess dietary vitamin E fed to rats over an extended period of time depressed their body weights.

The results of research on the effect of excess dietary vitamin E on the growth rate in animals vary widely. March et al. (1973) concluded that growth rate in chicks appeared to be relatively insensitive to excess dietary vitamin E (1,000 IU/kg diet), although a depression occurred with 2,200 IU vitamin E/kg diet in their short term study. Nockels et al. (1975) also reported that high levels of vitamin E supplementation (8,000 and 64,000 IU/kg diet) significantly reduced the chick body weight. However, McCuaig and Motzok (1970) fed a 10,000 IU vitamin E/kg diet to chicks and reported that growth rate was unaffected. Similar results have also been reported in the rabbit (Awad and Gilbreath, 1975). Effects of excess vitamin E on growth rate of rats have also been studied. In a 28 week study, Alfin-Slater et al. (1972) found there were no differences in weight gains of rats fed 100 mg vitamin E/day compared to those fed 30 mg vitamin E/day. However, Jenkins and Mitchell (1975) reported an increase in body weight of rats fed 600 and 6,000 IU vitamin E/kg diet for 2 months. Treatment with dietary vitamin E at 6,000 IU/kg diet (Jenkins and Mitchell, 1975) is a comparable level to an oral supplement

of 100 mg vitamin E/day (Alfin-Slater et al., 1972) if its assumed that the rat consumes 15 g diet/day. The reason for the wide discrepancy in results in this area remains to be investigated.

The organ weights (expressed as mg/100 g body weight) for rats fed different levels of vitamin E for 8 and 16 months are shown in Tables 3 and 4 respectively. For rats given dietary vitamin E from 25 to 25,000 IU/kg diet for 8 months, there were no significant differences among groups with respect to weights of liver, uterus, kidney and spleen. However, high levels of vitamin E significantly increased the groups relative heart weight after 8 months treatment. The regression analysis also revealed that after extending the treatment to 16 months, the groups fed excess vitamin E continued to have relative heart weights larger than those fed moderate levels of vitamin E. Also at this time the relative spleen weights were significantly increased. There were no significant differences among the rats fed different levels of vitamin E with respect to weights of liver, kidney and uterus. Hypervitaminosis E in rats has been reported to increase relative adrenal weight, but not effect relative weight of liver, kidney or spleen (Jenkins and Mitchell, 1975).

With the exception of the uterus, the relative organ weights in the vitamin E-free rats were significantly larger than those groups receiving vitamin E supplements for 8 months. This may be due to the depression in body weight after 3 to 4 months on the vitamin E-free diet. The weight loss represents massive muscle atrophy in vitamin E-free rats, with the organs not being affected as much during the same period of time. As a consequence, the relative sizes of the organs appear to be larger in vitamin E-free rats with the exception of the uterus.

## B. Hematological Parameters

There was no evidence in this study to suggest that excess vitamin E would lengthen the prothrombin time of rats. Instead, at the latter two test periods, 12 and 16 months, excess vitamin E actually resulted in decreased prothrombin times. These findings indicate that rats receiving adequate dietary vitamin K do not develop prolonged prothrombin time even when they are fed a very high level of vitamin E.

According to March et al. (1973), prothrombin time was lengthened in chicks fed excess amount of vitamin E. The prothrombin time was rapidly reversed by injection of vitamin K, which indicated an increase in the dietary requirement for vitamin K in the presence of excess dietary vitamin E. One previous study also reported that in some strains of rats the prothrombin level declined as higher doses of vitamin E were administered (Mellette and Leone, 1960).

It is difficult to compare the findings of March et al. (1973) with those of this study, however, because of the differences in the dietary requirement of vitamin K in rats and chickens. The induction of vitamin K deficiency is also affected by other physiological factors, such as the strain, age and sex of the experimental animal. Mellette and Leone (1960) have shown clear-cut differences between strains of rats and susceptibility to prolonged prothrombin time, as an indication of vitamin K deficiency. Also the female rat, as used in this study, is more resistant to vitamin K deficiency than the male rat (Johnson et al., 1960).

The results of this study, shown in Table 5, indicate that high levels of vitamin E for prolonged periods did not affect hemoglobin levels significantly. These findings are in agreement with the observation of Jenkins and Mitchells' (1975) in a short term experiment with rats.

Hematocrit values were not influenced by high dietary levels of vitamin E after 9 and 12 months of treatment (Table 6). However, after 16 months of treatment hematocrit values were significantly increased when vitamin E was fed at a level of 10,000 IU/kg diet or higher. March et al. (1973) have reported that hematocrit values were reduced in chicks fed 2,200 IU vitamin E/kg diet for 50 days. They observed that the reduction was more severe when the chicks were younger.

At present it is not possible to ascertain whether the hemopoietic system is influenced by an excess of vitamin E. Only recently has attention been centered on a possible role of vitamin E in heme and hemeprotein synthesis (Murty et al., 1970; Caasi et al., 1972; Nair, 1972). Other investigators, however, have not been able to show any involvement of vitamin E in heme synthesis (Carpenter, 1972; Diplock, 1974).

The results of the spontaneous hemolysis of erythrocyte in a buffered isotonic saline solution showed that only red blood cells of vitamin E deficient rats were susceptible to hemolysis. Excess dietary vitamin E did not alter the stability of the erythrocyte membrane to in vitro hemolysis.

#### C. Femoral Parameters

Bone composition of the rats in this experiment were not affected by high levels of vitamin E fed for 8 months. However, the presence of excess vitamin E in the diet for 16 months significantly (Table 10) reduced ( $P < 0.0005$ ) the ash content of bones. Treatment with increasing levels of dietary vitamin E, ranging from 250 to 25,000 IU/kg diet, increased the plasma alkaline phosphatase activity significantly. The altered alkaline phosphatase activities and ash content after 16 months may indicate increased turnover in bones of rats fed high levels of

vitamin E for prolonged periods.

March et al. (1973) reported that bone calcification was depressed when excess vitamin E (2,200 IU/kg diet) was administered to chicks fed either calcium-deficient or vitamin D-deficient diets. They concluded that excess vitamin E increased the requirement for vitamin D. There is considerable species variability in the dietary requirements of vitamin D, calcium and phosphorus. Animal performance depends on the absolute amounts of each nutrient as well as the relative amounts. Chickens require a higher calcium:phosphorus ratio than rats do for optimal growth. In rats, there is no extensive evidence to indicate that vitamin E is required for normal calcification when the dietary calcium and phosphorus are balanced and adequate (National Research Council, 1972). The National Research Council (1972) has recommended that approximately 1,000 IU vitamin D/kg diet be fed to growing rats. The vitamin D content in the experimental diet was 2,000 IU/kg diet. Thus, even if excess vitamin E has increased the requirement for vitamin D as March et al. (1973) have suggested in their work with chickens, this would not have been observed in this experiment since the animals received adequate levels of vitamin D with balanced levels of calcium and phosphorus.

#### D. Urinary Creatine and Creatinine

Urinary excretion of creatine and creatinine were apparently normal in all rats receiving high levels of dietary vitamin E for 11 months. Vitamin E-deficient rats had significantly higher creatine and lower creatinine levels in urine. Creatinuria is a recognized symptom associated with vitamin E deficiency. Hillman (1957) and Briggs (1974) have described creatinuria in three human subjects receiving large doses of vitamin E.

Briggs reported that an elevated serum creatine kinase accompanied the creatinuria. There was no indication in this study that excess vitamin E induces damage to skeletal muscle.

#### E. Fat Soluble Vitamins

##### 1. Liver and Plasma Vitamin E

The relationship between dietary levels of vitamin E and the storage of this vitamin in liver after 8 and 16 months treatment was linear when both values were expressed as logarithms (Figure 11). There was a significant deviation from the relationship between logarithm of vitamin E intake and log liver tocopherol concentration when the dietary level increased beyond 10,000 IU vitamin E/kg diet. As shown in Figure 11, further increases in dietary vitamin E had no significant effect on increasing the storage of this vitamin in liver. The total vitamin E content in the liver (Table 12) was approximately two-fold greater in rats treated for 16 months than those treated for 8 months. This accumulation of vitamin E was a result of both an increase in concentration of  $\alpha$ -tocopherol and enlargement of liver size as the experimental period was extended.

The findings of a linear relationship between increasing levels of vitamin E intake and liver levels when both were expressed in logarithmic units is supported by the work of Bolliger and Bolliger-Quaife (1956) and Wiss et al. (1962).

In this study the plasma tocopherol level increased significantly as the dietary vitamin E level was raised (Figure 12). The plasma tocopherol levels were not proportional to the vitamin E intake at all dietary levels, though. In addition, the plasma tocopherol levels were at least two-fold higher in the rats fed for 16 months, than those treated for 8 months.



According to Bolliger and Bolliger-Quaife (1956), Wiss et al. (1962) and Bieri (1972), there is a linear relationship between plasma tocopherol and the logarithm of the dose fed. The former two studies were short term experiments, while the report by Bieri (1972) was 25 weeks long and examined the effect of feeding a low level of vitamin E, 32 IU/kg diet. In the only long term study examining the effect of large doses of vitamin E on plasma tocopherol levels in rats, Alfin-Slater et al. (1972) reported that plasma tocopherol levels reflected the tocopherol level supplemented, but were not proportional to the dose.

## 2. Liver and Plasma Vitamin A

In this study, after 8 months treatment, the liver vitamin A storage of all vitamin E supplemented groups was significantly higher than of vitamin E-free groups. However, in the rats treated for 8 and 16 months, the change in dietary vitamin E ranging from 25 to 25,000 IU/kg diet showed no significant effect on altering liver vitamin A storage. The plasma vitamin A content of rats fed various dietary levels of vitamin E for 8 months was not tested, but those for 16 months were measured and no significant differences were observed between the groups. Therefore, it may be concluded that there was no interaction between high levels of dietary vitamin E and vitamin A in liver or plasma in this study.

Workers have confirmed that increased intakes of vitamin E increase the storage of vitamin A in the liver (Cawthorne et al., 1968; Prodouz and Navari, 1975). This vitamin E "sparing" effect on vitamin A has been shown at widely varying levels of vitamin E intake, for example, from 1 IU/week (Cawthorne et al., 1968) up to 6,000 IU/kg diet (Jenkins and Mitchell, 1975) have been reported to increase the liver vitamin A storage

in rats. Jenkins and Mitchell (1975) also reported that there was a significant increase in plasma vitamin A with increasing levels of vitamin E in the diet. The mechanism of action between these two vitamins is still unknown, but according to Cawthorne et al., (1968) the relationship between vitamin E and vitamin A in vivo cannot be regarded as that between an antioxidant and a peroxidizable substrate.

## F. Lipids

### 1. Liver Total Lipids and Cholesterol

Total lipids in liver were significantly increased by excess vitamin E supplementation (from 250 to 25,000 IU/kg diet) in rats treated for 8 months. Contrary to the results found after 8 months treatment, liver total lipid levels were not significantly altered among the rats treated for 16 months with different levels of vitamin E. No mechanism has been proposed to explain why excess vitamin E should increase liver total lipids only in younger rats. This cannot be accounted for by an increase in the level of liver cholesterol, because at both 8 and 16 months excess vitamin E did not significantly affect liver cholesterol concentration.

High dietary levels of vitamin E have been reported to increase the level of total lipids in liver (Alfin-Slater et al., 1972; Jenkins and Mitchell, 1975). Increasing dietary vitamin E intake also has been shown to enhance the development of alcohol induced fatty liver (Levander et al., 1973). Contrary to the above findings, other workers have reported that increasing dietary levels of vitamin E will decrease the level of total lipids in rat livers (Harrill et al., 1965; Harrill and Gifford, 1966; Prodouz and Navari, 1975). The levels of vitamin E used were much lower and the length of treatment was much shorter in these latter

investigations compared to the reports showing increases in the level of total lipids in liver.

## 2. Plasma Total Lipids and Cholesterol

The results of this study, shown in Figures 17 and 18, indicate that the plasma total lipids and cholesterol were not significantly altered following 8 months treatment with thigh levels of vitamin E. However, those rats treated for 16 months with high levels of dietary vitamin E (over 2,500 IU/kg diet) had significantly lower plasma total lipids and cholesterol. The regression curves of plasma total lipids on vitamin E were linear, while those on plasma cholesterol were not linear. Furthermore, the decrease in plasma total lipids was greater than that of plasma cholesterol in rats fed high levels of vitamin E suggesting that other components, such as triglycerides or phospholipids might also be affected.

It is difficult to compare the results of plasma total lipids and cholesterol in this study with those of other workers, since the level of vitamin E supplementation, the length of treatment and the dietary ingredients vary widely in the experiments. Vitamin E may play a role in altering plasma total lipid and cholesterol, but the results reported in the literature are inconsistent. It has been reported in numerous short term studies that high levels of dietary vitamin E will lower plasma cholesterol in rats (Chen et al., 1972; Harrill et al., 1965; Prodouz and Navari, 1975). However, other workers have reported that high dietary vitamin E had no effect on serum cholesterol in rats (Jenkins and Mitchell, 1975), rabbits (Awad and Gilbreath, 1975) and chicks (Koyangi et al., 1966).

## CHAPTER VI

## SUMMARY

The purpose of this study was to investigate the long-term effect of high levels of dietary vitamin E on various metabolic parameters in the rat. Six groups of female rats were fed for as long as 16 months the basal vitamin E-free diet with supplements ranging from 0 to 25,000 IU vitamin E (dl- $\alpha$ -tocopheryl acetate) per kilogram diet. The levels of vitamin E chosen were 0, 25, 250, 2,500, 10,000 and 25,000 IU/kg diet. All nutrients in the basal diet except vitamin E were adequate. The metabolic parameters studied in the rats fed excess vitamin E were compared statistically with the same parameters in rats receiving a moderate or normal level of dietary vitamin E.

The findings of this study on the long-term effect of excess intake of vitamin E in the rat were as follows:

- (1) Body weights were depressed in the groups fed 10,000 and 25,000 IU vitamin E/kg diet for 8 and 16 months.
- (2) High levels of dietary vitamin E increased the relative heart weight after 8 months and relative spleen weight after 16 months.
- (3) Hemoglobin values and spontaneous erythrocyte hemolysis were not influenced by excessive amounts of vitamin E. The prothrombin time was reduced after 12 months, while elevated hematocrit value was observed after 16 months of treatment.
- (4) The ash content of bone decreased with concurrent increase in plasma alkaline phosphatase activity after 16 months of treatment.

- (5) Urinary levels of creatine and creatinine were not affected by high levels of dietary vitamin E.
- (6) A logarithmic relationship was observed between dietary levels of vitamin E and the concentrations of this vitamin in liver and plasma.
- (7) The concentrations of vitamin A in liver and plasma were not affected by high levels of dietary vitamin E.
- (8) Total lipids in liver were significantly increased by excess vitamin E supplementation in rats fed for 8 months, but not in rats fed for 16 months.
- (9) Excess dietary vitamin E lowered plasma total lipids and cholesterol in rats treated for 16 months.

The results of this study suggest that excess vitamin E over prolonged periods of time have some harmful effects in rats.

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