EFFECT OF DIETARY SUPPLEMENTATION OF VITAMIN E
ON SERUM LIPIDS, LIPOPROTEINS AND
ATHEROGENESIS IN RABBITS FED A
CHOLESTEROLEMIC DIET

by

Mohan Viswanathan

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE
in the Department of
Kinesiology

MOHAN VISWANATHAN 1977

SIMON FRASER UNIVERSITY
September 1977

All rights reserved. This thesis may not be reproduced in whole or in part, by photocopy or other means, without permission of the author.
APPROVAL

Name: Mohan Viswanathan
Degree: Master of Science (Kinesiology)
Title of Thesis: Effect of vitamin E supplementation on
on serum lipids, lipoproteins and atherogenesis
in rabbits fed cholesterolemic diet.

Examining Committee:
Chairman: Dr. A. E. Chapman
Dr. N.M.G. Bhakthan
Senior Supervisor
Dr. R.A. Rockerbie
Dr. D B. Clement
Dr. M. Wertheim
External Examiner

Feb 13th 1978
CPC.
PARTIAL COPYRIGHT LICENSE

I hereby grant to Simon Fraser University the right to lend my thesis or dissertation (the title of which is shown below) to users of the Simon Fraser University Library, and to make partial or single copies only for such users or in response to a request from the library of any other university, or other educational institution, on its own behalf or for one of its users. I further agree that permission for multiple copying of this thesis for scholarly purposes may be granted by me or the Dean of Graduate Studies. It is understood that copying or publication of this thesis for financial gain shall not be allowed without my written permission.

Title of Thesis/Dissertation:
Effect of dietary supplementation of vitamin E on serum lipids, lipoproteins and atherogenesis in rabbits fed a cholesterolemic diet

Author:

(signature)

MUJISHWANATHAN

(name)

Dec 2, 1977

(date)
Abstract

Dietary supplementation of vitamin E in the treatment of coronary artery disease has remained a controversy for many years. No conclusive data is available regarding the protective nature of vitamin E supplementation against atherosclerosis by influencing serum lipoproteins. Therefore the following study was undertaken to determine the effect of vitamin E supplementation on serum cholesterol, triglycerides, phospholipids, lipoproteins and aortic lipid deposition in rabbits made hypercholesterolemic.

Twenty four male New Zealand white rabbits were divided into four groups of six animals each and they were maintained on the following diets for a period of twelve weeks.

Group N  Normal diet (Purina Rabbit Chow).
Group E  Normal diet + 11,000 I.U./kg $\alpha$-tocopherol.
Group C  Normal diet + 1 percent cholesterol.
Group CE Normal diet + 1 percent cholesterol + 11,000 I.U./kg $\alpha$-tocopherol.
While group E animals maintained a constant body weight throughout the study period, group CE animals gained 40 percent more weight than the other two groups. On biweekly analysis group CE animals had lower concentrations of serum cholesterol and triglycerides. The difference in lipids was mainly in the chylomicron remnants of d < 1.006. In addition nephelometric measurement of the lipoproteins revealed lower chylomicra concentration in the vitamin E supplemented animals. On gross examination of the thoracic aorta at the end of the twelve week dietary period, the development of lipid lesions was notably less in the supplemented group (CE). Although the total cholesterol in the arch and thoracic portion of the aorta did not differ between groups C and CE, the free cholesterol to cholesteryl ester ratio was significantly lower in group CE (0.56 for group CE as compared to 0.37 for group C). This difference in the ratio reveals that somehow vitamin E supplementation tends to maintain the aortic cholesterol composition closer to normal levels.

Indirect evidence for a possibly increased clearance of chylomicron remnants and decreased inhibition
of extrahepatic lipoprotein lipase as a result of vitamin E supplementation were obtained. A marked protective effect of vitamin E supplementation on atherogenesis in rabbits maintained on a hypercholesterolemic diet could not be demonstrated from the present investigation. However, a trend towards protection was evident from the parameters studied.
To

my parents

and

Dr. K. Krishna Pillai
Acknowledgement

I am deeply indebted to Dr. N. M. G. Bhakthan for all his help and supervision during the course of this investigation. Dr. R. A. Rockerbie showed sustained interest in this study for which I remain extremely grateful. My sincere thanks are due to Dr. D. Clement and Dr. A. K. Grover. Without their help the completion of this thesis would have been difficult. This study was in part supported by B.C. Heart Foundation grant in aid awarded to Dr. N. M. G. Bhakthan.
Table of contents

Introduction 1

Review of literature 5
  Metabolism of dietary cholesterol in rabbits 5
  Vitamin E and atherosclerosis 14

Materials and methods 17
  Experimental animals and diets 17
  Lipid and beta-lipoprotein determination 20
  Isolation of serum lipoproteins 22
  Chemical analysis of lipoproteins 23
  Extraction and determination of lipids in the aorta 24

Results 27
  Body weight 31
  Serum lipids 31
  Serum lipoprotein levels 47
  Chemical composition of lipoprotein groups 48
Aortic cholesterol  60

Discussion  66

Conclusions  76

Bibliography  78

Appendix  86
## List of tables

I. Grouping of test animals 18

II. Ultracentrifugal separation of lipoproteins 23

III. Body weight (x + s) in groups C, CE, N and E during the experimental dietary period of 12 weeks. 35

IV. Serum cholesterol concentration (x + s) in groups C, CE, N and E during the experimental dietary period of 12 weeks 40

V. Serum triglyceride concentration (x + s) in groups C, CE, N and E during the experimental dietary period of 12 weeks 44

VI. Serum phospholipid concentration (x + s) in groups C, CE, N and E during the experimental dietary period of 12 weeks 46
VII. Chylomicron concentration ($x + s$) in the serum of groups C and CE during the experimental dietary period of 12 weeks

VIII. Low density lipoprotein (LDL) concentration in the serum of groups C, CE, N and E during the experimental period of 12 weeks

IX. Chemical composition and levels of various lipoproteins in groups C, CE, N and E at 12 weeks

X. Quantities of cholesterol (mg/gm; $x + s$) and ratio of free cholesterol to cholesteryl ester in the aortic intima-media samples in animals supplemented with cholesterol (group C), cholesterol + vitamin E (group CE) vitamin E (group E) and control animals
List of figures

1. Species variation in the effect of cholesterol feeding on cholesterol metabolism (Dietschy and Wilson, 1970) 9

2. Aortae of rabbits supplemented with cholesterol (group C), cholesterol + vitamin E (group CE), vitamin E (group E) and control animals (group N) at 12 weeks 30

3. Body weights of animals supplemented with cholesterol (group C), cholesterol + vitamin E (group CE), vitamin E (group E) and control animals (group N) during the dietary period of 12 weeks 33
4. Effect of supplementation of cholesterol (group C) and cholesterol + vitamin E (group CE) on serum cholesterol concentration during the dietary period of 12 weeks

5. Effect of supplementation of cholesterol (group C) and cholesterol + vitamin E (group CE) on serum triglyceride concentration during the dietary period of 12 weeks

6. Effect of supplementation of cholesterol (group C) and cholesterol + vitamin E (group CE) on chylomicron concentration during the dietary period of 12 weeks

7. Effect of supplementation of cholesterol (group C) and cholesterol + vitamin E (group CE) on serum LDL concentration during the dietary period of 12 weeks
8. Quantities of cholesterol in the aortic intima-media preparations in animals supplemented with cholesterol (group C), cholesterol + vitamin E (group CE), vitamin E (group E) and control animals (group N) at 12 weeks
Atherosclerosis is an arterial disease that has attracted much attention in recent years as the chief cause of death in many parts of the world. The specific factors which cause this disease or its genesis has not yet been fully established. Plasma lipoproteins are thought to play an important role quantitatively and physiologically in the pathogenesis of atherosclerosis. Therefore the study of the metabolism of plasma lipoproteins is of great importance.

Plasma lipoproteins are complex macromolecules whose function is to solubilise the otherwise insoluble lipids and to transport them from their organs of synthesis to their sites of utilization (Levy, 1976). They are generally separated into four families according to their physical characteristics and chemical composition. Ultracentrifugal separation on the basis of their density, classifies them as chylomicra, very low density lipoproteins (VLDL), intermediate density lipoproteins (IDL), low density lipoproteins (LDL) and high density lipoproteins (HDL).
Cholesterol and its esters are the principal lipids found in atherosclerotic lesions (Adams, 1971), and the source of these lipids have been identified as low density lipoproteins in humans (Smith and Slater, 1970; 1972; Hoff et al, 1975). There is increasing evidence that plasma lipoprotein levels are closely related to atherosclerosis in humans and experimental animals.

Cholesterolemia, hyperlipoproteinemia and atherosclerosis can be induced in a variety of animals by feeding them cholesterol-rich diets (Kritchevsky, 1963; Clarkson, 1971). The rabbit has been used extensively for many years in studies of atherosclerosis. The normal rabbit has plasma lipoprotein classes and apolipoproteins that are comparable to those of man (Mills and Tylaur, 1971; Shore et al, 1974; Shore and Shore, 1975). Since apolipoproteins are the major determinants of lipoprotein structure and metabolism, it can be expected that mechanisms for synthesis and catabolism of the lipoproteins will be similar in the rabbit and man (Shore and Shore, 1976). Furthermore, rabbits respond rapidly to changes in dietary cholesterol and provide adequate blood and tissue samples for biochemical studies. It is, therefore, a useful animal for investigating lipoprotein metabolism and to study the effects of agents that could influence lipoprotein metabolism.
For the past thirty years the efficacy of using vitamin E supplementation as a protective agent against coronary artery disease has remained a controversy. Claims made in support of such an action have been based on uncontrolled clinical studies and so have often been questioned by medical scientists (Hodges, 1973).

No attempt has yet been made to see whether high levels of vitamin E supplementation to rabbits maintained on a hypercholesterolemic diet could have a protective action against atherosclerosis by influencing serum lipoproteins. The present study is an attempt to answer the following questions.

Does vitamin E supplementation in the diet of rabbits maintained on a hypercholesterolemic diet for a period of twelve weeks—

a. change the levels of serum lipids and lipoproteins?

b. prevent the changes in the composition of lipoproteins induced by hypercholesterolemia?

c. alter the amount and nature of cholesterol deposition in the aortic wall?
The rational behind conducting this study is expected to emerge from the review of pertinent literature in the next chapter.
Chapter 2

Metabolism of dietary cholesterol in rabbits

Although different species of animals respond to cholesterol feeding in varying ways the basic characteristics of cholesterol biosynthesis are identical in all animals studied to date. Small differences in metabolism are caused by variations in absorption or excretion or both. Figure 1 represents four typical cases of cholesterol metabolism (Dietschy and Wilson, 1970). Case 1 represents the situation in all species during low cholesterol intake. Cholesterol synthesis accounts for almost all of the cholesterol entering the system and this is balanced by an equal excretion of neutral and acidic sterols. In man (case 2), only a limited amount of cholesterol can be absorbed and this amount is compensated for by inhibition of hepatic cholesterol synthesis. The miscible pool of cholesterol rises, while excretion and degradation remains constant during cholesterol intake.
Rats and dogs have the capacity to absorb larger quantities of cholesterol than can be compensated for by inhibition of synthesis. However, cholesterol-enhanced bile acid formation helps to maintain a steady concentration which is only slightly raised (Dietschy and Wilson, 1970). The rabbit absorbs cholesterol more efficiently than humans (Connor and Lin, 1974; Ross and Zilversmit, 1976; Massaro and Zilversmit, 1977), and rats (Zilversmit and Hughes, 1974). Moreover, hepatic enzymes that convert cholesterol to bile acids appear to be less efficient in this species (Hellstrom, 1965). Until recently, it was thought that the rabbit cannot increase the conversion of cholesterol to bile acids as a compensatory mechanism in the maintenance of plasma cholesterol concentration as is the case in the cholesterol-fed rat (Wilson, 1964), and dog (Abell et al., 1956). Sex and strain related variations in the plasma cholesterol concentration in normal and cholesterol fed rabbits have been reported (Filios and Mann, 1956; Laird et al., 1970; Adams et al., 1972; Roberts et al., 1974). Massaro and Zilversmit (1977) studied the sterol kinetics in normal and cholesterol-fed rabbits from two strains and found that increased bile acid excretion is a protective mechanism responsible for the relatively low plasma cholesterol concentration found in some cholesterol-fed rabbits. This increased bile acid excretion
Fig. 1.  Species variation in the effect of cholesterol feeding on cholesterol metabolism (Dietschy and Wilson, 1970).
Cholesterol Synthesis

Cholesterol Absorption

Fecal Neutral Sterol Excretion

Fecal Acidic Sterol Excretion

Cholesterol Synthesis

Cholesterol Absorption

Fecal Neutral Sterol Excretion

Fecal Acidic Sterol Excretion

Cholesterol Synthesis

Cholesterol Absorption

Fecal Neutral Sterol Excretion

Fecal Acidic Sterol Excretion

Cholesterol Synthesis

Cholesterol Absorption

Fecal Neutral Sterol Excretion

Fecal Acidic Sterol Excretion
could clear only a fraction of the cholesterol that the rabbit absorbs and hence the accumulation of cholesterol and a subsequent rise in plasma cholesterol concentration occurs.

Since cholesterol is transported in plasma in the form of lipoproteins it is desirable to consider lipoprotein metabolism in the cholesterol-fed rabbit. It has been known for many years that feeding cholesterol-enriched diets to rabbits rapidly results in increased concentrations of plasma very low density lipoproteins (VLDL). A large proportion of the VLDL is found to be esterified cholesterol with only relatively little triglyceride (Garlick and Courtice, 1962; Camejo et al, 1973). A high percentage of the increased serum cholesterol resulting from cholesterol-enriched diets can be located in the VLDL fraction (Courtice and Garlick, 1962; Fraser et al, 1976).

The process of metabolism of this class of lipoprotein in the cholesterol-fed rabbit has been given due attention only in the recent past. The metabolic origin of VLDL in the cholesterol-fed rabbit has been a matter of active discussion and two major sources of hypercholesterolemic VLDL have been considered. Shore et al (1974) noticed that hypercholesterolemic VLDL are larger
than normal VLDL, and have a different apolipoprotein distribution. They concluded that hypercholesterolemic VLDL are not degradation products of normal triglyceride-rich plasma VLDL and suggested a hepatic origin for those particles. Evidence seems to be growing in favour of the alternative possibility that these lipoproteins are formed as a result of the degradation of triglyceride rich intestinal lipoproteins. Increases in chylomicron esterified cholesterol relative to triglyceride are known to result from partial degradation of chylomicra by hepatic tissues in vivo (Redgrave, 1970; Mjos et al, 1975), or from exposure of chylomicra to the action of lipoprotein lipase in vitro (Nilson and Akesson, 1975). Camejo et al (1974) noted that VLDL in hypercholesterolemic rabbit serum is a broad spectrum of comparatively large particles, rich in cholesterol, and possibly representing 'remnant' chylomicra. Rose (1972) showed the cholesteryl esters of hypercholesterolemic rabbits were not derived from the action of lecithin cholesterol acyl transferase on free cholesterol in the plasma. In addition the presence of 'remnants' or 'ghosts' of chylomicra in serum has been demonstrated by a number of workers (Nestel et al, 1963; Redgrave, 1970). Ross and Zilversmit (1977) employed a double labelling technique that discriminated lipoproteins containing cholesteryl esters of dietary origin from
hepatogenous lipoproteins, to study the origin of VLDL in cholesterol-fed rabbits. They concluded from their findings that nearly two-thirds of cholesteryl esters in VLDL of cholesterolemic rabbit plasma are primarily intestinal products. This study confirms the suggestions made by French et al (1955) that the lipemia of rabbits fed cholesterol could be attributed primarily to failure in disposition of the cholesterol containing residues of the lymph chylomicra.

The possibility that the rabbit has a defective 'remnant removal mechanism' is a hypothesis that is getting acclamation currently. Redgrave (1973), from experiments on the removal from circulation of chylomicra radioactively-labelled in their cholesterol and triglyceride moieties, has shown the uptake of chylomicron 'remnants' by the liver to be slower in normal rabbits than in normal rats, and especially slow in hypercholesterolemic rabbits. Another study by Rodriguez et al (1976) looked at the clearance of 125 I-labelled VLDL from normal and hypercholesterolemic rabbits. The rate of clearance of hypercholesterolemic VLDL by the normal and cholesterol fed rabbits was found to be similar and slower than the clearance of normal VLDL. They concluded that the delayed clearance of hypercholesterolemic VLDL may not be due to a defective remnant removal, but
rather due to structural changes in the cholesterol-rich VLDL. It is worth mentioning that in addition to the structural changes reported ( Camejo et al, 1973; 1974), an arginine-rich apolipoprotein is found to be one of the major apolipoproteins of hypercholesterolemic VLDL (Shore et al, 1974). Whether it is this compositional change that causes the delay in its clearance is not yet known. The hepatic uptake of these particles in rabbits was studied by Ross and Zilversmit (1977). The normal rabbits cleared VLDL from the plasma rapidly through the liver, while the hypercholesterolemic rabbits showed a delayed hepatic uptake. Therefore it was revealed that the hypercholesterolemic VLDL circulates for a much longer period in the plasma of the cholesterol-fed rabbit. One of the mechanisms proposed by the authors deserves attention. They suggest that chylomicron remnants accumulate due to saturation of the removal mechanism when the rate of dietary cholesterol input exceeds the normal maximal clearance rate and that saturation could occur at the level of conversion of hepatic cholesterol to bile acids, or at the hepatic receptor for remnants.
The disorder in humans that closely resembles hypercholesterolemia in rabbits is type III hyperlipoproteinemia (Fraser et al., 1972). It was proposed that the primary defect in type III hyperlipoproteinemia is an impaired catabolism of VLDL to LDL and that it shows a defective remnant removal system (Hazzard and Bierman, 1971). Another recent study has confirmed the above suggestion, and shows that this defect could be corrected by oestrogen therapy (Chait et al., 1977).

The atherogenicity of VLDL and chylomicron remnants has not been studied carefully. Remnant lipoprotein particles produced in vitro were seen to be taken up but poorly catabolised by rat aortic smooth muscle cells growing in cell culture (Bierman et al., 1973). This finding along with the recent proposals by Zilversmit (1973) and Hulsmann et al. (1975) that the remnant formation on the arterial endothelial surface could initiate aortic lesions deserves further study.
Vitamin E and atherosclerosis

There seems to be no agreement in the literature regarding the role of vitamin E as a protective agent against atherosclerosis. Claims that large doses of vitamin E might benefit patients with coronary artery disease have been largely based on clinical reports and case histories which lacked consistency and statistical analysis of data and so have been questioned by medical scientists (Hodges, 1973). Studies on experimental animals seem to suggest an involvement of vitamin E in atherosclerosis. Sulkin and Sulkin (1970) found that in rats maintained on a vitamin E free diet for 280-450 days, intimal lesions and fibrotic plaques developed in the aorta. The formation of these lesions and plaques was accelerated by the dietary addition of fifteen to twenty per cent vitamin E free hydrogenated cottonseed oil. The addition of two percent cholesterol, however, had no effect on the lesions which contained no cholesterol or sudanophilic lipid. It was reported that daily supplements of vitamin E prevented the development of the intimal changes. There is recent evidence that vitamin E and A supplementation in the diets of rabbits fed a hypercholesterolemic diet markedly reduced lipid
infiltration into the aorta along with a slight decrease in the level of plasma cholesterol (Brattsand, 1975). In addition vitamin E deficiency in rabbits was found to produce significantly high aortic lipid deposition and a non-significantly higher cholesterol content (Awad and Gilbreath, 1975).

Most of the studies which try to relate vitamin E and atherosclerosis seem to accept the atherogenic role of lipid-peroxides. Since vitamin E is considered to be an antioxidant it is believed to prevent lipid peroxidation in tissues and in turn to have a protective action against atherosclerosis. Glavind et al (1952) have shown that atherosclerotic aortae contain lipoperoxides and that the peroxide content parallels the degree of severity of the atherosclerosis, while normal aortae are free from lipoperoxides. The high values of Glavind et al for the peroxide content of aortic lipids was later found to be due to artifactual formation of peroxides during tissue preparation and extraction of lipids (Woodford et al, 1965). However, this finding does not exclude the possibility that lipid peroxides play a role in atherosclerotic process. A study indicating increased lipid peroxidation in the aortae of rats fed a cholesterol supplemented diet supports the assumption of the involvement of peroxides in atherogenesis.
(Tsai, 1975). Alpha-tocopherol concentrations in the aortic tissue from cholesterol fed rabbits have been studied. Total tocopherol concentration was lower in aortic tissue displaying a high level of atheroma (Kirk, 1973). Most tocopherol enters the blood-stream via lymph where it is associated with chylomicrons and very low density lipoproteins (Bierii and Farrell, 1976). After entering the blood, the tocopherol in chylomicra rapidly equilibrates with the other plasma lipoproteins (Peake et al, 1972). There is one report on the possibility of a protective action of vitamin E against cholesterol induced atherosclerosis in rabbits (Iwakami, 1965). The vitamin E was administered to the rabbits through intramuscular injections rather than by way of dietary supplementation. A significantly lowered occurrence of lesions was noticed in the aortae of the rabbits supplemented with vitamin E in this study. No data on serum lipid or aortic lipid were reported.
Chapter 3

Materials and methods

Experimental animals and diets

Twenty four male New Zealand white rabbits weighing 2.4 to 3.6 kg were housed individually in wire-bottomed cages. Their approximate age was ten weeks.

Eighteen of the animals were fed for a period of forty days with powdered Purina rabbit chow (PRC) mixed with enough water to constitute it in the form of a dough. This period allowed the animals to learn to eat a semisolid food which was the form of the experimental diets and to adjust to the new surroundings. The groups of animals and their respective diets are shown in Table I.
Table I.

Grouping of test animals

<table>
<thead>
<tr>
<th>Group</th>
<th>Diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>Purina Rabbit Chow (PRC)</td>
</tr>
<tr>
<td>E</td>
<td>PRC + 11,000 I.U./kg ( \alpha )-tocopherol</td>
</tr>
<tr>
<td>C</td>
<td>PRC + 1% cholesterol</td>
</tr>
<tr>
<td>CE</td>
<td>PRC + 1% cholesterol + 11,000 I.U./kg ( \alpha )-tocopherol.</td>
</tr>
</tbody>
</table>

Diets containing \( \alpha \)-tocopherol and / or cholesterol were prepared in the laboratory. Appropriate amounts of dl \( \alpha \)-tocopherol (Calbiochem, San Diego, CA, USA) and / or cholesterol (ICN Pharmaceuticals, Cleveland, Ohio, USA) were mixed thoroughly into the dry pulverised PRC, and further mixed with enough water to make it acceptable to the animals.
Each of the rabbits were given one hundred and twenty grams of food daily. The animals consumed this amount within two to three hours. Water was provided ad libitum.

A period of one week was allowed between starting each group of animals on the prescribed diet. This time gap provided enough time to obtain and process samples collected from the animals during and at the end of the experimental dietary period.

The four groups of animals were maintained on the experimental diets for a period of twelve weeks and their body weights were monitored during this period.

Animals were bled from the ear vein every two weeks after an overnight fast, about seven ml of blood was collected from each rabbit and the serum was separated. Serum total cholesterol, triglycerides, phospholipids and beta-lipoproteins were estimated from these samples.
At the end of the dietary period, the animals were subjected to ether anaesthesia, the abdominal cavity was opened, and blood was collected from the inferior vena cava. The thoracic aorta from the thoracic arch to the diaphragm was dissected and removed into Ringer's solution. It was slit open and rinsed free of blood. After taking photographs of the luminal surface of the aorta, the intimal and medial layers were carefully dissected out from the entire aorta according to the method described by Wolinsky and Daly (1970). This method was helpful in separating the layers. The pieces of intima-media sections were blotted dry, weighed and immediately frozen in liquid nitrogen and stored at $-10^\circ$C. The different classes of lipoproteins were separated from the serum and their composition was analysed. The quantities of free and esterified cholesterol in aortic intima and media were also determined.

Lipid and beta-lipoprotein determination

Serum total cholesterol and triglycerides were estimated by a commercial laboratory*. The automated ferric chloride method of Levine and Zak (1964) was used to estimate total cholesterol.

*B.C. Biomedical Laboratory
Serum triglycerides were estimated according to the enzymatic procedure of Bucolo and David (1973) adapted to the GEMSAEC centrifugal analyzer (Chong-Kit and Mc Laughlin, 1974) and based on the measurement of liberated glycerol as indicated by the following reactions.

\[
\text{Glycerol} + \text{ATP} \rightarrow \text{Glycerol phosphate} + \text{ADP}.
\]

\[
\text{ADP} + \text{Phosphoenol pyruvate} \rightarrow \text{ATP} + \text{Pyruvate}.
\]

\[
\text{Pyruvate} + \text{NADH} \rightarrow \text{Lactate} + \text{NAD}.
\]

The amount of glycerol present is proportional to the decrease in absorbance of NADH at 340 nm and is the basis for the calculation of triglyceride content.

The phosphomolybdate method of Zilversmit and Davis (1950) was used to determine serum phospholipids photometrically at 660 nm in a Beckman DB - GT spectrophotometer.
A turbidimetric method was used to determine the levels of chylomicra and LDL in the serum (Sholnick et al, 1972). This method is based on the selective precipitation of lipoproteins by sulfated polysaccharides in the presence of divalent cations varying in concentrations. Used as precipitants were 0.025 M calcium chloride in normal saline, 0.1 M magnesium chloride in 0.7 per cent sodium chloride and 0.1 M magnesium chloride in 1 per cent sodium chloride. The turbidity was measured at 650 nm using a Beckman DB-GT spectrophotometer.

Isolation of serum lipoproteins

Serum from animals of each group were pooled for lipoprotein fractionation by a standard floatation technique (Havel and et al, 1955). Ultracentrifugation was performed in a Beckman model L5-40 ultracentrifuge, using a 30.2 rotor at 30,000 RPM. Four density classes were separated as stated in table II.
Table II.

Ultracentrifugal separation of lipoproteins

<table>
<thead>
<tr>
<th>Density</th>
<th>Lipoprotein</th>
<th>Centrifugation time</th>
</tr>
</thead>
<tbody>
<tr>
<td>d &lt; 1.006</td>
<td>VLDL</td>
<td>24 hours at 10 C</td>
</tr>
<tr>
<td>d 1.006 - 1.019</td>
<td>IDL</td>
<td>24 hours at 10 C</td>
</tr>
<tr>
<td>d 1.019 - 1.063</td>
<td>LDL</td>
<td>24 hours at 10 C</td>
</tr>
<tr>
<td>d 1.063 - 1.21</td>
<td>HDL</td>
<td>48 hours at 10 C</td>
</tr>
</tbody>
</table>

Chemical analysis of lipoproteins

After dialysis against 0.15 M saline containing 0.01 per cent ethylenediaminetetraacetate (EDTA), total cholesterol, triglycerides, phospholipids and protein content of each lipoprotein fraction was estimated. Total cholesterol and phospholipids were measured according to the methods previously described. Triglycerides were measured using Sigma reagents and a procedure based on the methods of Kessler and Lederer (1965) and Fletcher (1968). Triglycerides were extracted with isopropanol and the
interfering substances were removed by a solid adsorbant. The triglyceride containing extract was then subjected to the following reactions.

Triglycerides + KOH \longrightarrow \text{Glycerol + Fatty acids.}

Glycerol + Periodate \longrightarrow \text{Formaldehyde.}

Formaldehyde + NH\textsubscript{4} + Acetylacetone \longrightarrow \text{Diacetyldihydro-lutidine.}

The absorbance of the final yellow product was measured at 410 nm and related to triglyceride concentration.

For the estimation of protein, lipoprotein fractions were delipidated with chloroform-methanol (1:1) according to the method of Camejo et al (1974). The apoproteins thus obtained were assayed using the method of Lowry et al (1951) with bovine serum albumin as the standard.

Extraction and determination of lipids from aorta

The wet weight of the intima-media preparations ranged from 0.2 mg to 0.6 mg. They were extracted in ten ml of chloroform-methanol (2:1) and the extracts were washed according to the method of Folch et al (1957). The washed
extract was taken to dryness under nitrogen, and redissolved in one ml of chloroform. Cholesterol and cholesteryl esters were separated by thin layer chromatography (TLC) on 5 20 cm Polygram silica G precoated plastic sheets (Brinkman Instruments Inc, N.Y.) and eluted with chloroform - methanol (3:1). Free and esterified cholesterol in these eluates was estimated using a sensitive fluorometric technique (Bondjers and Bjorkerud, 1971) in a Baird Atomic spectrofluorometer. Slight modifications were introduced to the original method. ten ml sealed glass ampoules were used instead of glass-stoppered test-tubes for incubation of the samples at 60 C. The final volumes were adjusted to four ml instead of one ml. These modifications gave consistently reproducible results.

Treatment of data

Time dependence of changes in the various parameters was determined by fitting a hyperbolic function between each individual parameter and time. The curve fitting problem was solved by fitting weighted least square lines between the reciprocal values of the parameters as well as time. A line was fitted for each group for an individual parameter and intercept of the line determined.
The corresponding intercepts of the lines for different groups were used to test the level of significance \((p = 0.95)\) in the differences between the groups. The parameter aortic cholesterol was determined only at the end of the dietary period, and its values were directly used to conduct \(t\)-tests.

A listing of Fortran IV computer program is given in the Appendix. The program was run on IBM 370.
Chapter 4

Results

All of the experimental animals survived the dietary period. None of the group N and group E animals showed any peculiarity in their feeding habits or deterioration in health.

Two animals from group C, after four weeks on the experimental diet, declined to consume all of the food given to them daily. This phenomenon would last for intervals of two or three days after which the animals would revert to a normal eating habit. This behaviour was found to persist until the end of the experimental period. One animal from this group developed xanthoma on both of the hind legs after six weeks on the cholesterol diet, and for the last four weeks, ate only very little of the food given. There was only very little fat deposit around the internal organs in this animal at the time of sacrifice and the aorta had lipid lesions. The serum was greenish yellow in this animal and the liver was very pale and contained greenish fluid which
was thought to be bile. The lipid and lipoprotein data from this animal was discarded because of these reasons. All of the animals from group C were noticed to produce a reduced quantity of urine daily. Neither the urine volumes nor the water consumption could be measured in any of the animals since this was not included in the experimental design.

Group CE animals appeared healthy and showed no noticeable changes in their feeding habits until the final week of the experimental period. Two of the animals showed loss of appetite for two days, but were normal afterwards.

At the time of sacrifice after twelve weeks on the experimental diet, none of the aortae from groups N and E had any abnormalities in their morphology. In group C, all of the animals had lipid lesions in the region of the aortic arch. The thoracic portion of the aortae of four animals had lipid lesions of varying degrees (Fig 2). Of the aortae from two animals left in that group, one had small lipid lesions around the intercostal arteries, while the other was free from lesions.

The aortic arches of group CE animals were also affected, but the distribution of lipid lesions in this region was found to be sparse. The thoracic portion of the
Fig. 2. Aortae of rabbits supplemented with cholesterol (group C), cholesterol+ vitamin E (group CE), vitamin E (group E) and control animals (group N) at 12 weeks.
aortae from two animals had lipid lesions, while each of the remaining four animals had only little lipid deposits around some of the intercostal arteries.

The aortae of three of the animals from group C were found to have tougher walls. It is possible that these aortae had more fibrous connective tissue in them. This was not noticed in any of the aortae from group CE.

Body weight

At the beginning of the study, the average body weight between the groups of rabbits ranged from 2.9 to 2.98 kg (Table 3). A significant gain in weight (p < 0.05) was noticed in group CE animals as compared to the other three groups (Fig. 3). Of the four groups, the vitamin E supplemented animals (group E) gained the least weight, though not significantly less than group N. The trend in weight gain was very similar in groups N and C.

Serum lipids

Serum cholesterol, triglyceride and phospholipid levels were significantly higher in both the cholesterol fed
Fig. 3. Body weights of animals supplemented with cholesterol (group C), cholesterol + vitamin E (group CE), vitamin E (group E) and control animals (group N) during the dietary period of 12 weeks.
Table III. Body weight ($x \pm s$) in groups C, CE, N and E during the experimental dietary period of 12 weeks.
<table>
<thead>
<tr>
<th>Group</th>
<th>Body weight (kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>C</td>
<td>2.98±0.19</td>
</tr>
<tr>
<td>CE</td>
<td>2.93±0.28</td>
</tr>
<tr>
<td>N</td>
<td>2.93±0.23</td>
</tr>
<tr>
<td>E</td>
<td>2.9±0.47</td>
</tr>
</tbody>
</table>
groups C and CE than in their control groups N and E. These serum lipid concentrations were found to remain constant in both the control groups N and E, during the investigation.

At the beginning of the test period, serum cholesterol levels varied between 44 and 68 mg/dl in the four experimental groups (Table IV). While the control groups N and E maintained approximately the same low concentration until the end of the dietary period, both the cholesterol fed groups showed a tremendous increase in their serum cholesterol levels. Serum cholesterol concentrations rose more sharply in the non-vitamin E supplemented group (C), as compared to the supplemented group CE (Fig. 4). Compared to group CE, the rate of increase in the serum cholesterol level (Fig. 4) was significantly higher in group C (p < 0.05). A mean serum cholesterol level of 2064 mg/dl was seen in group C at the end of the study period whereas only 1748 mg/dl was noticed in group CE.

Serum triglyceride concentrations ranged from 58 mg/dl to 67 mg/dl in the four groups of animals at the start of the study (Table V). Groups N and E showed a more or less similar level of serum triglyceride throughout the study period. Group C animals tended to show a steady rise in their serum triglyceride levels which was statistically
Fig. 4. Effect of supplementation of cholesterol (group C) and cholesterol + vitamin E (group CE) on serum cholesterol concentration during the dietary period of 12 weeks.
Table IV. Serum cholesterol concentration ($x \pm s$) in groups C, CE, N and E during the experimental dietary period of 12 weeks.
<table>
<thead>
<tr>
<th>Group</th>
<th>Serum Cholesterol (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>C</td>
<td>47±10.7</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>CE</td>
<td>58±8.7</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>N</td>
<td>43.7±4.3</td>
</tr>
<tr>
<td>E</td>
<td>67.8±26.2</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Fig. 5. Effect of supplementation of cholesterol (group C) and cholesterol + vitamin E (group CE) on serum triglyceride concentration during the dietary period of 12 weeks.
Table V. Serum triglyceride concentration (x ± s) in groups C, CE, N and E during the experimental dietary period of 12 weeks.
<table>
<thead>
<tr>
<th>Group</th>
<th>Serum Triglycerides (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>C</td>
<td>66.7±2.22</td>
</tr>
<tr>
<td>CE</td>
<td>58±8.7</td>
</tr>
<tr>
<td>N</td>
<td>62.3±12.9</td>
</tr>
<tr>
<td>E</td>
<td>62.7±11.6</td>
</tr>
</tbody>
</table>
Table VI. Serum phospholipid concentration 
\((x \pm s)\) in groups C, CE, N and E during 
the experimental dietary period of 12 
weeks.
<table>
<thead>
<tr>
<th>Group</th>
<th>Serum Phospholipids (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>C</td>
<td>116.5±18.9</td>
</tr>
<tr>
<td>CE</td>
<td>126.8±25.3</td>
</tr>
<tr>
<td>N</td>
<td>95.8±12.8</td>
</tr>
<tr>
<td>E</td>
<td>117.8±14.5</td>
</tr>
</tbody>
</table>
different from that in group CE ($p < 0.05$) which maintained relatively lower levels during the twelve weeks (Fig. 5). At the end of the experimental period group C showed a serum triglyceride level of 337 mg/dl and group CE had a level of only 179 mg/dl. Variation in the serum triglyceride values between the animals was noticed to be less in group CE as compared to group C.

Serum phospholipids showed control values of 96 to 126 mg/dl at the beginning and in groups N and E during the twelve week dietary period (Table VI). Even though groups C and CE showed a rise in the serum phospholipids, and reached final concentrations of 459 mg/dl and 521 mg/dl respectively, the increases in the values were not significantly different between the two groups.

Serum lipoprotein levels

Chylomicra were absent in the serum of fasting rabbits at the beginning of the study and during the experimental period in groups N and E. Cholesterol feeding markedly raised chylomicron concentration in the serum of both groups C and CE (Table VII). Values showed high individual variation between the animals, especially among animals in group C. Group C and CE showed an increase in
serum chylomicra levels with increasing time on the cholesterol diet. There was a trend for the group C animals to show higher levels of serum chylomicra than that of group CE (Fig. 6). This difference was not statistically significant, mainly due to the high individual variation in chylomicron levels.

The initial level of LDL ranged from 0.04 to 0.183 absorbance units in the four groups of animals. An even lower level was maintained in groups N and E during the studied. Serum LDL level rose to 1.02 absorbance units in group C while in group CE it rose to a level of 1.49 units after two weeks of cholesterol feeding (Table VIII). In group C the LDL level dropped during the remaining ten weeks on the experimental diet whereas group CE animals seemed to maintain a steadier level, closer to that after 2 weeks on the cholesterol diet (Fig. 7). However, this difference in the level of LDL between the two groups was not statistically significant.

Chemical composition of lipoprotein groups

Lipid and protein content in the beta-lipoprotein fractions from both of the cholesterol fed groups were elevated when compared to that of the control animals.
Fig. 6. Effect of supplementation of cholesterol (group C) and cholesterol + vitamin E (group CE) on chylomicra concentration during the dietary period of 12 weeks.
Table VII. Chylomicron concentration ($x \pm s$) in the serum of groups C and CE during the experimental dietary period of 12 weeks.
<table>
<thead>
<tr>
<th>Group</th>
<th>0</th>
<th>2</th>
<th>4</th>
<th>6</th>
<th>8</th>
<th>10</th>
<th>12 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>5.11±</td>
<td>0.94</td>
</tr>
<tr>
<td>C</td>
<td>0</td>
<td>2.22±</td>
<td>3.8±</td>
<td>3.85</td>
<td>1.84</td>
<td>1.32±</td>
<td>1.32±</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.7</td>
<td></td>
<td></td>
<td></td>
<td>0.85</td>
<td>0.85</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.99</td>
<td>0.99</td>
</tr>
<tr>
<td>CE</td>
<td>0</td>
<td>0.653±</td>
<td>1.32±</td>
<td>2.47±</td>
<td>2.47±</td>
<td>1.42</td>
<td>1.42</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.51</td>
<td></td>
<td></td>
<td></td>
<td>0.58</td>
<td>0.58</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.35</td>
<td>1.35</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.98</td>
<td>1.98</td>
</tr>
</tbody>
</table>
Fig. 7. Effect of supplementation of cholesterol (group C) and cholesterol + vitamin E (group CE) on serum LDL concentration during the dietary period of 12 weeks.
Table VIII. Low density lipoprotein (LDL) concentration (x ± s) in the serum of groups C, CE, N and E during the experimental dietary period of 12 weeks.
<table>
<thead>
<tr>
<th>Group</th>
<th>Low density lipoproteins (absorbance 650 nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>C</td>
<td>0.18 ± 0.168</td>
</tr>
<tr>
<td>CE</td>
<td>0.178 ± 0.063</td>
</tr>
<tr>
<td>N</td>
<td>0.04 ± 0.023</td>
</tr>
<tr>
<td>E</td>
<td>0.176 ± 0.134</td>
</tr>
</tbody>
</table>
Very low density lipoproteins (VLDL) from group C had distinctly higher levels of total cholesterol (1760 mg/dl), triglyceride (174 mg/dl) and phospholipid (220 mg/dl) as compared to the fraction from group CE, where the lipid content was as follows: cholesterol 1060 mg/dl, triglyceride 38 mg/dl, phospholipid 167 mg/dl (Table IX). The difference in the triglyceride content of the VLDL between these two groups was conspicuous. Protein content was almost the same in both the groups. The total serum VLDL level was 60 percent higher in group C when compared to that in group CE (Table IX).

The noticeable difference in composition in the intermediate density lipoproteins (IDL) fraction between groups C and CE was in the triglyceride content. IDL from group C contained 96.8 mg/dl triglyceride while group CE IDL had only 38.7 mg/dl. There was a higher level of phospholipid in group C IDL compared to group CE IDL. But protein and cholesterol content were slightly more in the group CE IDL. The composition of IDL in the control animals were not different between the groups. Total IDL level did not show any profound differences between groups C and CE.

Differences in cholesterol, triglyceride and protein content of LDL were noticed between groups C and CE.
Table IX. Chemical composition and levels of various lipoproteins in groups C, CE, N and E at 12 weeks.
<table>
<thead>
<tr>
<th>Lipoprotein</th>
<th>Group</th>
<th>Cholesterol (mg/dl)</th>
<th>Triglycerides (mg/dl)</th>
<th>Phospholipid (mg/dl)</th>
<th>Protein (mg/dl)</th>
<th>Serum Lipoprotein level</th>
</tr>
</thead>
<tbody>
<tr>
<td>VLDL</td>
<td>CE</td>
<td>1760.0</td>
<td>174.2</td>
<td>220</td>
<td>38.2</td>
<td>2192.4</td>
</tr>
<tr>
<td>d &lt; 1.006</td>
<td>N</td>
<td>1060.0</td>
<td>38.7</td>
<td>167.5</td>
<td>39.0</td>
<td>1305.2</td>
</tr>
<tr>
<td></td>
<td>E</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IDL</td>
<td>CE</td>
<td>244.0</td>
<td>96.8</td>
<td>83.5</td>
<td>11.8</td>
<td>436.1</td>
</tr>
<tr>
<td>d 1.006-</td>
<td>N</td>
<td>302.0</td>
<td>38.7</td>
<td>70.0</td>
<td>18.8</td>
<td>429.5</td>
</tr>
<tr>
<td>1.019</td>
<td>E</td>
<td>26.0</td>
<td>43.6</td>
<td>2.0</td>
<td>5.0</td>
<td>76.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LDL</td>
<td>CE</td>
<td>200.0</td>
<td>58.1</td>
<td>82.5</td>
<td>20.6</td>
<td>361.2</td>
</tr>
<tr>
<td>d 1.019-</td>
<td>N</td>
<td>296.0</td>
<td>38.7</td>
<td>82.5</td>
<td>57.6</td>
<td>424.8</td>
</tr>
<tr>
<td>1.063</td>
<td>E</td>
<td>24.0</td>
<td>14.5</td>
<td>2.0</td>
<td>0.8</td>
<td>41.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HDL</td>
<td>CE</td>
<td>32.0</td>
<td>58.1</td>
<td>2.0</td>
<td>20.6</td>
<td>112.7</td>
</tr>
<tr>
<td>d 1.063-</td>
<td>N</td>
<td>32.0</td>
<td>58.1</td>
<td>2.0</td>
<td>57.6</td>
<td>149.7</td>
</tr>
<tr>
<td>1.21</td>
<td>E</td>
<td>28.0</td>
<td>38.7</td>
<td>42.5</td>
<td>68.8</td>
<td>179.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
While group C LDL contained 58.1 mg/dl triglyceride, group CE LDL had a level of 38.7 mg/dl. Group CE LDL had slightly more cholesterol and protein than group C LDL, and this was reflected in the total LDL level.

No difference in the cholesterol composition of HDL was evident between the cholesterol fed and control groups of animals. Triglyceride and phospholipid content were similar in group C HDL and CE HDL. Phospholipid was drastically reduced in the HDL fraction from the cholesterol fed groups, C and CE. While protein content of group CE HDL was almost the same as that of the control HDL, group C HDL had only half the amount of protein. This caused the total level of HDL from group C to be slightly lower than that from the other three groups.

Aortic cholesterol

Cholesterol content in the aortic intima-media sections from the cholesterol treated groups of animals C and CE were significantly higher than that in the animals from control groups N and E (Fig. 8). The amount of total cholesterol did not differ between groups C and CE (Table X). The level of free cholesterol tended to be slightly
raised in aortae from group CE and the level of cholesteryl ester was lowered. The ratio of free cholesterol to cholesteryl ester in the aortae of group CE animals was closer to that in the control animals. This ratio was different in group C (0.374) as compared to that in group CE (0.562), where there was more cholesteryl ester and less free cholesterol in the aortae. The difference in the ratio of free cholesterol to cholesteryl ester between groups C and CE was significant at the 0.1 level.
Fig. 8. Quantities of cholesterol in the aortic intima-media preparations in animals supplemented with cholesterol (group C), cholesterol + vitamin E (group CE), vitamin E (group E) and control animals (group N) at 12 weeks.
Table X. Quantities of cholesterol (mg/gm; x ± s) and ratio of free cholesterol to cholesteryl ester in the aortic intima-media samples in animals supplemented with cholesterol (group C), cholesterol + vitamin E (group CE), vitamin E (group E) and control animals (group N) at 12 weeks.
<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Total Cholesterol</th>
<th>Free Cholesterol</th>
<th>Cholesteryl Ester</th>
<th>Free Cholesterol/Cholesteryl ester</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>5</td>
<td>36.34 ±</td>
<td>9.89 ±</td>
<td>26.46 ±</td>
<td>0.374 ±</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10.18</td>
<td>4.06</td>
<td>6.99</td>
<td>0.1</td>
</tr>
<tr>
<td>CE</td>
<td>5</td>
<td>34.08 ±</td>
<td>12.18 ±</td>
<td>21.9 ±</td>
<td>0.562 ±</td>
</tr>
<tr>
<td></td>
<td></td>
<td>14.15</td>
<td>6.21</td>
<td>8.56</td>
<td>0.23</td>
</tr>
<tr>
<td>N</td>
<td>5</td>
<td>2.31 ±</td>
<td>0.81 ±</td>
<td>1.49 ±</td>
<td>0.67 ±</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.98</td>
<td>0.2</td>
<td>0.88</td>
<td>0.31</td>
</tr>
<tr>
<td>E</td>
<td>3</td>
<td>2.12 ±</td>
<td>0.89 ±</td>
<td>1.23 ±</td>
<td>0.76 ±</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.57</td>
<td>0.57</td>
<td>0.86</td>
<td>0.07</td>
</tr>
</tbody>
</table>
Chapter 5

Discussion

The present study is believed to be the first to reveal a possible influence of vitamin E on body weight gain, serum lipids and lipoproteins in rabbits maintained on a hypercholesterolemic diet.

There have been reports of reduced thyroid function and inhibition of growth in young rats and chicken, as a result of vitamin E supplementation (Valenti and Bottarelli, 1965; March et al, 1973). A rather similar condition was observed in this investigation where vitamin E supplemented animals showed a decrease in weight gain during the twelve weeks as compared to the control group (N). This decrease was not statistically significant. Whether this was due to an altered thyroid activity as in rats and chicken is not known.
Animals fed a diet supplemented with vitamin E and cholesterol (group CE), showed an increased weight gain when compared to the other groups. The reason for this remains obscure. In a study where hyperlipidemic patients were treated with clofibrate and a supplementation of vitamin E, two out of fourteen subjects showed excessive weight gain (Vessby et al, 1977). Whether their weight gain was due to vitamin E supplementation is not clear from the presented data.

As observed by a number of investigators (Connor and Lin, 1974; Ross and Zilversmit, 1976; Massaro and Zilversmit, 1977), one percent cholesterol diet raised the level of serum lipids in cholesterol-fed rabbits (groups C and CE. (Table IV). The rise in serum lipids was noticeable after two weeks on the cholesterol diet. A serum triglyceride rise in cholesterol-fed rabbits has been reported earlier and a mechanism has been proposed to explain this phenomenon (Huang et al, 1970). Huang et al (1970) looked at the mechanism of triglyceridemia in rabbits fed a high cholesterol-diet. They concluded that the presence of cholesterol in high amounts in the substrate acted upon by lipoprotein lipase competitively inhibits the enzyme activity, and that this was the cause of hypertriglyceridemia in cholesterol-fed rabbits. Another
study has pointed out the inhibition of human lipoprotein lipase (LPL) activity by unesterified cholesterol (Fielding, 1970).

Serum chylomicron levels also were raised and followed a trend similar to the rise in serum cholesterol in groups C and CE (Table VII). The raised amount of cholesterol and triglycerides in group C could be located in the very low density lipoproteins (VLDL) fraction isolated at the end of the twelve week dietary period. This finding agrees with that of Fraser et al (1976), Redgrave et al (1976) and Ross and Zilversmit (1977) who established that the major portion of the dietary cholesterol remains in VLDL of cholesterol-fed rabbits for a very lengthy time.

Interesting was the finding that the rise in serum cholesterol and triglyceride levels was significantly lower in group CE as compared to group C, while both groups were consuming a diet containing equal amount of cholesterol. It is seen in this study that the lowering of cholesterol in group CE was mainly in the serum VLDL fraction (Table IX). At the same time, the difference in the serum triglycerides was due to its lowered level in VLDL, intermediate density lipoproteins (IDL) and low density lipoproteins (LDL). The decrease in the triglyceride content of VLDL in group CE was
very conspicuous (Table IX). In short it is evident that IDL and LDL in group CE have slightly more cholesterol distributed in them with markedly less cholesterol in VLDL. The reverse is the case with beta-lipoproteins of group C. This group had more cholesterol in the VLDL fraction with less cholesterol in IDL and LDL (Table IX). This data along with the data on the triglyceride content of the beta-lipoproteins leads to two possible interpretations.

1. An increased chylomicron remnant clearance may have taken place in group CE animals. This would account for its lowered serum cholesterol level which was found to be due to lowered VLDL cholesterol.

2. There could be a reduced inhibition of extrahepatic lipoprotein lipase (LPL) which was reflected in decreased serum triglyceride levels and also in VLDL triglyceride content as shown in table IX. A tendency for the group CE animals to maintain a higher level of LDL in contrast to the condition in group C (Fig. 7) also supports such a hypothesis (Nichols et al, 1968). An evidence from the present study to support the possibility of a reduced inhibition of LPL is the increased level of high density lipoproteins (HDL) found in group CE animals compared to that of group C animals (Table IX). Increases in HDL as a
result of lipoytic degradation of VLDL have been reported (Levy et al., 1966; Nichols et al., 1968).

It is quite possible that the two separate possibilities mentioned are interrelated. Since lipoprotein lipase is found to be inhibited by high cholesterol levels in VLDL (Huang et al., 1970), the lowered VLDL cholesterol content found in group CE could be one of the reasons for reduced inhibition of this enzyme. Moreover, free cholesterol is found to inhibit LPL more than does cholesteryl ester (Fielding, 1970; Huang et al., 1970). It would therefore be interesting to determine whether VLDL in group CE contained more esterified cholesterol and less free cholesterol which would bring about a similar effect. Even though the possibility of an increased hepatic clearance of chylomicron remnants in group CE animals is not proven in this study, the existence of a more efficiently functioning liver in group CE animals compared to that of group C animals is conceivable.

The human condition that closely resembles hypercholesterolemia in rabbits is type III hyperlipoproteinemia (Fraser et al., 1972). Type III hyperlipoproteinemia, although uncommon is important because it is often associated with accelerated peripheral and
possibly coronary atherosclerosis (Morganroth et al, 1975; Slack, 1975). The primary defect in this abnormality has been shown to be an impaired catabolism of VLDL to LDL and a defective chylomicron remnant removal (Hazzard and Bierman, 1971; Chait et al, 1977). On the basis of the present study, it would be worthwhile to study the efficacy of using vitamin E supplementation in patients suffering from type III hyperlipoproteinemia to alter their lipoprotein profiles.

The group C animals were found to have reduced urine output in contrast to the other three groups of animals. There is only one report in the literature describing such a phenomenon in cholesterol-fed rabbits (Massaro and Zilversmit, 1977). No explanation could be found to account for this observation. Because of the possibility that the animals on cholesterol diets had reduced urinary outputs the presence of impaired renal function should not be excluded. Alterations in normal renal function are found to cause abnormalities in lipoprotein metabolism in humans (Lewis et al, 1966; Akamatsu, 1977; Bagdade and Albers, 1977). These studies also report a reduced level of plasma high density lipoproteins (HDL) in patients with chronic renal failure.
Whether a similar case could account for the changes in HDL level in rabbits in group C is open to speculation. However, the change in the level of HDL between groups C and CE was mainly due to a difference in protein content and not due to the lipid in them (Table IX). This raises the question whether it was the functional apoprotein C or the structural apoprotein A which differed in quantity between the HDL in animals from groups C and CE. Apoprotein C is considered to be an activator of extrahepatic lipoprotein lipase and one of the major roles of plasma HDL in the metabolic pathway of lipoproteins is to serve as a flexible 'reservoir' of apoprotein C subunits (Eisenberg and Levy, 1976). Transfer of apoprotein C from HDL to chylomicra during alimentary chylomicronemia has been demonstrated (Havel et al, 1973). Later when chylomicron triglycerides are cleared from the plasma compartment, the activator protein transfers back to HDL. In a case where there is inhibition of LPL activity it is perhaps logical to assume that the activator protein apoprotein C may not be readily available to be transferred to newly formed chylomicra. This could also result in a decreased LPL activity which would further aggravate the situation of chylomicron metabolism in the cholesterol-fed rabbit. Whether vitamin E supplementation effectively protected group CE animals from a possible impairment of renal function is a matter of conjecture.
The results from this study are inadequate to substantiate the two proposed possible mechanisms for reduced serum cholesterol and triglyceride levels. These results do however suggest these possibilities. In order to elucidate the action of vitamin E supplementation on VLDL metabolism in hypercholesterolemic rabbits, the hepatic clearance of labelled VLDL and activity of extrahepatic LPL should be studied in animals under similar dietary conditions.

The results from this study indicated that the protective effect of vitamin E supplementation was not very pronounced on atherogenesis in the aortae of rabbits maintained on a one percent cholesterol diet. From their gross appearance only moderate differences between groups C and CE could be noticed in the distribution of lipid lesions in the region of the aortic arch. A more marked difference was seen in the density of the lesions in the thoracic portion of the aortae of these animals. While four aortae from group C were affected in the thoracic portion, only two were seriously affected in the animals of group CE.
This difference was not reflected in the total cholesterol content in the aortic tissue from the arch and thoracic portions. In retrospect it appears that if the aortic arch and the thoracic portions were separated before the analysis of deposited cholesterol, more profound changes might have been noticed in the thoracic portion of the aortae.

Eventhough the total cholesterol deposition in the aortae from groups C and CE did not differ, there was a noticeable difference in the ratio of free cholesterol to cholesteryl esters (Table 8). The ratio was significantly different ($p < 0.1$) in group C from that of group CE. It is not clear from this study what the factors were that decreased the cholesteryl esters and increased the free cholesterol in the aortae in group CE animals. The increased amount of free cholesterol in the aortae of group CE animals, could be considered favourable to the animals because free cholesterol has been demonstrated to be readily diffusible through cellular membranes (Rothblat et al, 1967).
Two of the possible ways of explaining the above mentioned finding are decreased esterification of free cholesterol in the aortic wall or an increased activity of cholesteryl esterase which breaks down cholesteryl esters. It has been shown that hypercholesterolemia promotes esterification of free cholesterol in the aortic tissue in vitro (St. Clair et al, 1970; 1977) and in rabbit aorta in vivo (Day and Proudlock, 1974). These studies report that cholesterol esterification occurs prior to the appearance of grossly visible atherosclerotic lesions. During atherogenesis, newly esterified cholesteryl esters accumulate in the aorta (St. Clair et al, 1968; 1970). A deficiency in the lysosomal cholesteryl ester hydrolase has been proposed as one of the mechanisms to explain this phenomenon (Peters et al, 1973). Whether any of these changes are responsible for the difference noticed in the ratio of free cholesterol to cholesteryl esters in aortic intima-media sections from animals of groups C and CE remains to be investigated.

Changes brought about in the aortae by feeding a cholesterol-diet may have differed in animals in group C and CE at earlier periods than the present twelve weeks. It would be worthwhile to concentrate more on this aspect in future studies.
Chapter 6

Conclusions

From the present investigation, a marked protective effect of vitamin E supplementation on atherogenesis in rabbits maintained on a hypercholesterolemic diet could not be demonstrated. However, a trend towards protection emerges from the parameters studied. Indirect evidence for increased clearance of chylomicron remnants and a decreased inhibition of lipoprotein lipase as a result of vitamin E supplementation were obtained. Studies on VLDL metabolism and lipoprotein lipase activity in animals maintained under similar conditions may yield information which would validate the claims made based on the results from this study.

Studies on growth and thyroid function of rabbits supplemented with high level of vitamin E would also be relevant. Another aspect that was found interesting during the present study was the decrease in the urinary output of animals fed a high-cholesterol diet. Scant attention seems to have been paid in the past to this effect of a
In this investigation certain of the statistical analyses showed only low levels of significance due to the relatively high variation between individual animals in each group. A dose response study using different levels of vitamin E and cholesterol supplementation using a larger population of animals might be of additional value as a follow-up study. Such an investigation would indicate an optimum dose level that may be employed without inducing unwanted side effects. Agents that are effective in accelerating VLDL removal through an increase in lipoprotein lipase activity are of much importance and vitamin E appears to exert a similar activity in the hypercholesterolemic rabbits. It is possible to make this claim on the basis of indirect evidence provided by the lowered serum lipids and lipoproteins in animals supplemented with cholesterol and vitamin E.
Bibliography


COURTICE, F.C., GARLICK, D.G. The permeability of the capillary wall to the different plasma lipoproteins of the hypercholesterolemic rabbit in relation to the their size. Quart. J. Exp. Physiol. 47: 221-227, 1962.


MORGANROTH, J., LEVY, R.I., FREDRIKSON, D.S. Annals of Internal Medicine, 82, 158, 1975.


STACK, J. Postgraduate Medicine, 51 (suppl. 8): 27, 1975.


//FORTRAN EXEC PORTGCLG
//FORTRAN SYSIN DD *

DIMENSION T(50), P(50), SUBIN(50), VELIN(50)

C PROGRAM TO FIT PROBABLE LEAST SQUARE LINE INTO
C 1/Y = A+B/X

C INPUT PARAMETERS-
C CARD 2- NUMBER OF POINTS IN FIRST DATASET, I2
C CARD 1 - NUMBER OF DATASETS , FORMAT I2
C CARD 3- FIRST X AND Y, FFORMAT 2F10.4
C CARD 4,5,------SUBSEQUENT X AND Y OF FIRST SET
C NEXT CARD STARTS NEXT DATA SET TO BE GIVEN IN
C THE SAME MANNER AS THE FIRST SET
C GO AHEAD GIVE ALL THE PROMISED NUMBER OF DATA SETS
C

C OUTPUT PARAMETERS-
C X, Y, YCALCULATED, 1/X, 1/Y, SLOPE, INTERCEPT
C STANDARD DEVIATIONS IN SLOPE, INTERCEPT,
C 1/Y, Y.

READ(5,10) M
10 FORMAT(I2)
DO 1000 MEMBER=1,M
READ(5,10) N
SUMX=0.0
SUMY=0.0
SUMXX=0.0
POWER=0.0
SUMXY=0.0
DO 200 J =1,N
READ(5,50) T(J),P(J)
50 FORMAT(2F10.4)
SUBIN(J)=1.0/T(J)
VELIN(J)=1.0/P(J)
W=P(J)**2
SUMX=SUMX+SUBIN(J)*W
SUMXX=SUMXX+(SUBIN(J)**2)*W
SUMY=SUMY+VELIN(J)*W
SUMXY=SUMXY+SUBIN(J)*VELIN(J)*W
POWER=POWER+W
200 CONTINUE
CUTNUM=SUMY*SUMXX-SUMX*SUMXY
SNUM=POWER*SUMXY-SUMX*SUMY
DENOMI=POWER*SUMXX-SUMX**2
CUTSAT=CUTNUM/DENOMI
WRITE(6,250) MEMBER,N
SLOPES=SNUM/DENOMI
250 FORMAT(1H1,20X,7HDATASET,2X,I2,20X,I2,2X,6HPOINTS)
WRITE (6,270)
270 FORMAT(//'11X,4HTIME,10X,5HVVALUE,5X,10HCALC VALUE,'\n18X,3H1/T,15X,3H1/V)
WRITE (6,280)
280 FORMAT(/)
SUMREC=0.0
SUMVAL=0.0
DO 600 J=1,N
VELCAL=CUTSAT+SLOPES*SUBIN (J)
SUBCAL=(VELIN (J)-CUTSAT)/SLOPES
PCAL=1.0/VELCAL
WRITE (6,400) T(J) ,P(J) ,PCAL,SUBIN (J) ,VELIN (J)
400 FORMAT(5(3X,F12.5))
DIFVAL=P(J)-PCAL
DIFREC=VELCAL-VELIN (J)
SUMREC=SUMREC+DIFREC*DIFREC
SUMVAL=SUMVAL+DIFVAL*DIFVAL
600 CONTINUE
VARREC=SUMREC/N
VARVAL=SUMVAL/N
STAREC=SQRT(VARREC)
STAVAL=SQRT(VARVAL)
WRITE (6,620) SLOPES,CUTSAT
620 FORMAT(//'5X,6HSLOPE=,F12.5,10X,10HINTERCEPT=,F12.5)
WRITE (6,650)
650  FORMAT(/20X,19HSTANDARD DEVIATIONS)
    WRITE(6,700) STAREC,STAVAL
700  FORMAT(/,5X,19HSD IN INVERSE OF Y=,F12.5,10X,8HSD IN Y=,F12.5)
    SUM=0.0
    DO 800 J=1,N
        SUM=SUM+VELIN(J)
800  CONTINUE
    COVARI=STAREC*N/SUM
    ERRINT=CUTSAT*COVARI
    WRITE(6,850) ERRINT
850  FORMAT(/5X,28HEXPECTED ERROR IN INTERCEPT=,F12.5)
1000 CONTINUE
    DUMMY=0.0
    STOP
END
//GO.SYSIN DD *