CHANGES IN PLASMA LIPIDS OF TURKEYS FOLLOWING THE ADDITION OF DRIED EGG OR WHEY PROTEIN TO THE DIET

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PREFACE

Much personel satisfaction has been gained from this endeavor. This study not only has given me the opportunity to pursue my interest in physiology, but also has given me a greater appreciation for the amount of work involved in scientific research. The pursuit of this project has not been without difficulty. Often it seemed that there would not be enough time to finish. However, truly rewarding acccomplishments are never obtained without adversities.

Completion of this work would not have been possible without the guidance and counseling of my advisor, Dr. Calvin G. Beames Jr. I am grateful for his advice and continuous encouragement. He introduced me to physiology and sparked my interest for a career in medicine. I can honestly say that I have benefitted from being his student and friend.

I would like to express my appreciation to Dr. Duane L. Garner and Dr. John A. Bantle for their suggestions and time spent reviewing this manuscript. I especially would like to thank Dr. Bantle for his support with my teaching responsibilities.

Lastly, I must thank my wife, Kris. She has not only given her loving support, but has actually participated in much of the work contained in this study. She is my inspiration.

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CHAPTER I

INTRODUCTION

Importance of Study

Much attention has been given recently to the study of the influence of dietary factors upon plasma cholesterol levels. This is justifiably so since the leading cause of death now in the U. S. is coronary heart disease and other diseases associated with atherosclerosis (Levy, 1981). These conditions have been directly linked to the level of cholesterol in one's plasma (Rifkind, 1984). Large amounts of evidence have established that the higher the plasma total or low density lipoprotein cholesterol level, the greater the risk that coronary heart disease will develop. This evidence has focused attention on reductions in plasma cholesterol levels by various means, including drug and diet therapy.

As a result of this link between high levels of plasma cholesterol and the increased risk of coronary heart disease, modifications in diets have been recommended by groups such as the American Heart Association and the United States Senate Select Committee on Nutrition and Human Needs (1977). The recommendations made by the Senate committee

call for reductions in the intake of foods high in saturated fats and cholesterol such as whole milk, milk products, and eggs. While it is true that these foods do contain relatively large amounts of cholesterol and fats, the scientific community still must question the extent to which these foods raise the cholesterol levels in plasma. Contrary to the diet recommendations, investigations have revealed that milk and eggs increase one's plasma cholesterol much less than originally assumed. In fact, intense interest has been generated in the possibility of some protective factor in milk that actually reduces serum cholesterol.

It is not disputed that purified cholesterol added to the diet raises plasma cholesterol levels. However, the presence of purified cholesterol in diets of test animals is not a natural component. Other compounds that are present in cholseterol-rich foods are excluded when purified crystalline cholesterol is added to the diet. The exclusion of these compounds removes their influence upon cholesterol digestion and absorption. Therefore, data from studies where crystalline cholesterol was added to the diet may be misleading.

Purpose and Objectives

The intent of this study is two fold. First, it is designed to determine the effect of dietary whey protein on

plasma cholesterol profiles, thus testing to see if whey protein is that portion of milk that contains the hypocholesterolemic factor. Second, it is designed to compare the hypercholesterolemic effect of dietary cholesterol from two sources, namely dried eggs and purified crystalline cholesterol.

CHAPTER II

REVIEW OF LITERATURE

Diet's Influence on Plasma Cholesterol

An individual's plasma cholesterol levels are influenced by a number of different factors including, body metabolism, genetic make up, level of exercise, certain hormones, as well as diet. When the relationship between plasma cholesterol and heart disease was first recognized several years ago, the treatment of choice of most clinicians for hypercholesterolemia was to drastically lower the intake of foods high in saturated fats and cholesterol. However, only some ten percent of the cholesterol in plasma is derived from the diet (Rivers, 1977). Scientists and clinicians soon came to realize that the level of cholesterol in one's plasma is affected by a great many factors, and that a single dietary treatment approach does not prove to be effective in reducing the risk of coronary heart disease (Mann, 1977).

The Milk Factor

Previous studies have indicated that milk and certain

milk products contain some factor that reduces plasma cholesterol levels (Hepner, 1979; Howard, 1977). Initial studies on a hypocholesterolemic factor in milk were carried out by Mann (1964) on a tribe in Eastern Africa called the Maasai. Each Maasai tribesman ordinarily consumes four to five liters of fermented whole milk per day in addition to substantial quantities of meat. In spite of this diet high in saturated fat and cholesterol, the Maasai have low serum cholesterol levels and a very low incidence of clinical coronary heart disease. Mann suggested in subsequent investigations that the hypocholesterolemic factor in fermented milk may be 3-hydroxy-3-methyl-glutaric acid (HMG) (Mann, 1974; Mann, 1977). This compound is a known inhibitor of the enzyme 3-hydroxy-3-methyl-glutaryl CoA reductase (HMG CoA reductase). This enzyme catalyzes the rate limiting step in the biosynthesis of cholesterol from acetate. However, no analytical data has been presented to indicate the presence of HMG in fermented milk.

Other workers have suggested that orotic acid, 6-carboxy-2,4-dioxypyrimidine, may be the compound in milk that reduces plasma cholesterol levels (Boguslawski, 1974; Kelly, 1970). Orotic acid is found commonly in bovine milk at an average concentration of 73 milligrams per liter (Webb, 1974). In vitro studies by Bernstein (1977) indicated that orotic acid reduced the biosynthesis of cholesterol in rat liver homogenates and they suggest that orotic acid decreases plasma cholesterol by inhibiting the

synthesis of cholesterol in the liver. However, Okonkwo (1974) showed that orotic acid added to a basal diet at 0.15% resulted in grossly increased liver size. This was due to the accumulation of various lipids in the liver, most notably cholesterol. This might suggest a shift of cholesterol from the plasma to the liver. If this is the case, orotic acid would certainly not be the hypocholesterolemic factor in milk since fatty livers have not been reported in studies where individuals have consumed as much as five liters of fermented milk per day.

The Turkey as an Experimental Subject

The rat is commonly used for studying the effects of dietary factors on plasma cholesterol levels. However, rats do not develop coronary heart disease. An animal model of choice should develop atherosclerotic lesions resembling those seen in humans. The use of avian species for the study of atherosclerosis has been well documented (Shih, 1983). The turkey has been used as an experimental model and has proven useful in studies involving cardiovascular diseases. Critical and unique features of the turkey are the spontaneous and age related development of atherosclerosis. These animals develop hypertension (Krista, 1970) and are susceptible to aortic aneurysm (Clarkson, 1959). Some nicholas strain large breasted white turkeys are hypertensive beginning at eight weeks of age

(Schoffner, 1977). Simpson and Harms (1969) discovered that atherosclerosis could be induced in cholesterol-fed turkeys after a short period of time. All of these characteristics of the turkey provide the experimenter with a valuable model for studying atherosclerotic parameters.

Cholesterol

Cholesterol is a very important molecule in living cells. It is the precursor of steroid hormones, bile acids, and is a major constituent of cell membranes.

Since cholesterol is a lipid and hydrophobic, it must be carried in plasma as soluble packages covered with proteins. These packages are known as lipoproteins. Lipoproteins are categorized by their densities. The higher the lipid to protein ratio the lower the density of the lipoprotein (Table I). These density classes include, chylomicrons, very low density lipoproteins (VLDL), low density lipoproteins (LDL), and high density lipoproteins (HDL). Each density class serves to transport lipids via blood to certain target sites. The different protein species of each lipoprotein direct it to its proper destination. Figure one gives a diagramatic representation of lipoprotein function in the blood.

The least dense lipoproteins, chylomicrons, pick up fats that are derived from food in the small intestine. They consist of up to 95% triglycerides, and very little

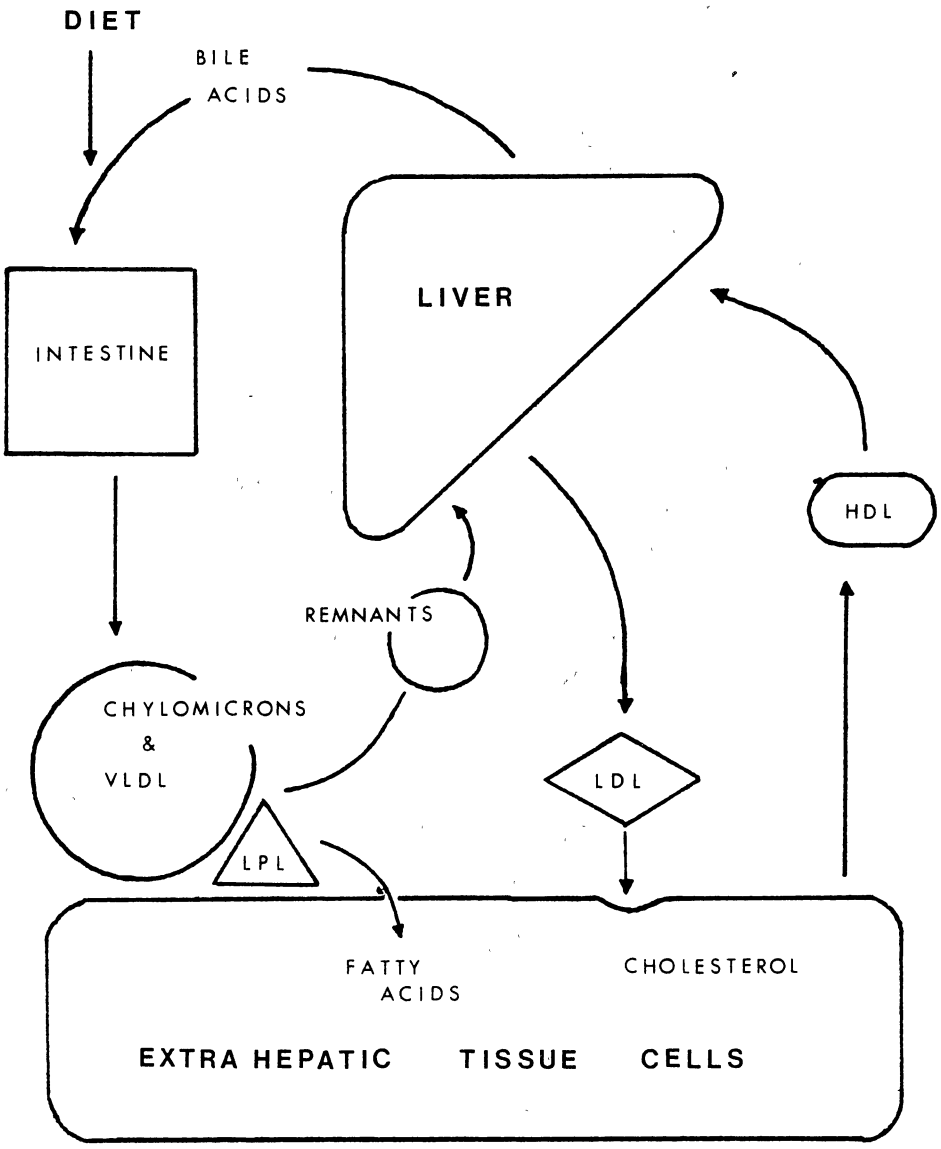
TABLE I

COMPOSITION OF HUMAN LIPOPROTEIN
(percent dry weight)

Density Class	TG	Chol.	CE	PL	Prot.	Density (mg/dl)
Chylm.	86	1	5	7	2	0.95-0.97
VLDL	50	7	13	20	10	0.95-1.006
LDL	8	10	30	30	22	1.006-1.063
HDL	8	4	12	24	52	1.063-1.21

Adapted from Schonfeld (1979)
Abbreviations: VLDL, very low density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein; TG, triglyceride; Chol., cholesterol; CE, cholesterol ester; PL, phospholipid; Prot., protein

Figure 1. Simplified illustration of lipid transport and fate of lipoproteins in human plasma. Dietary triglycerides and cholesterol enter the cells of the small intestine where they are assembled into water soluble particles known as Chylomicrons and Very low density lipoproteins (VLDL). These triglyceride rich lipoproteins enter the blood via the lymph system and deliver triglycerides to the peripheral tissues. Lipoprotein Lipase (LPL), present on luminal surfaces of vascular endothelium, hydrolyzes the triglyceride into free fatty acid and glycerol. As they lose their triglycerides, chylomicrons and VLDL become progressively smaller to form Remnants; which are taken up by the liver. The liver synthesizes cholesterol-rich Low density lipoproteins (LDL) which serve to transport cholesterol to peripheral tissues. Cholesterol is removed from extra-hepatic cells and transported to the liver by High density lipoprotein (HDL). Adapted from Schonfeld (1979).


DIET
BILE
ACIDS
INTESTINE
LIVER
HDL
REMNANTS
CHYLOMICRONS
&
VLDL
LDL
LPL
FATTY
ACIDS
CHOLESTEROL
EXTRA HEPATIC TISSUE CELLS

cholesterol. The next lightest lipoproteins, VLDL, carry about 15% of the total cholesterol in plasma and serve a similar function as chylomicrons, that is to transport absorbed fats from the small intestine to the tissues of the body. Both VLDL and chylomicrons deliver their triglycerides to the peripheral tissues where they are hydrolyzed by an endothelial bound enzyme called lipoprotein lipase. Thereby, free fatty acids and glycerol become available for uptake and utilization by peripheral tissues. As chylomicrons lose their triglycerides and glycerol they become progressively less dense and form particles known as remnants. Since much of the triglyceride is removed, these remnants differ from the original chylomicrons and VLDL in that they now contain a higher concentration of cholesterol esters. These remnants then are taken up by the liver and are thought to be converted to LDL. How this conversion is carried out is not known.

The LDLs are rich in cholesterol and transport approximately 65% of the cholesterol in blood. Their role is to transport cholesterol to peripheral tissues and regulate the synthesis of cholesterol in these peripheral cells. Generally, cells outside the liver obtain cholesterol from the plasma rather than by synthesizing it on their own. It is this lipoprotein that has been implicated as the major source of cholesterol in the plaques of atherosclerotic arteries.

The mechanism for removal of LDL cholesterol has been

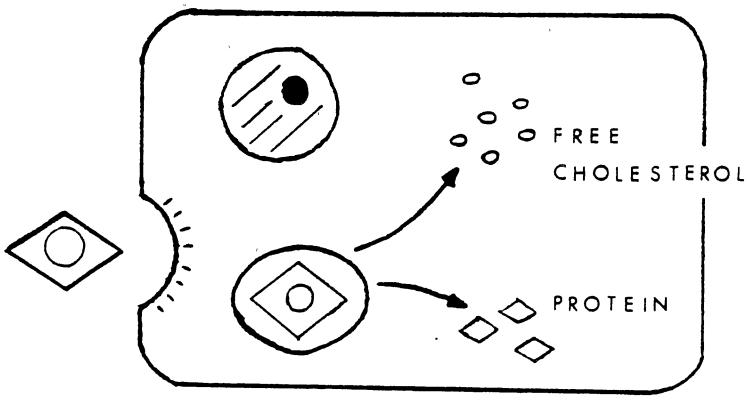
well documented (Brown, 1981), and is illustrated in figure 2. Extrahepatic cells contain LDL receptors on their membranes. These receptors, known as coated pits, have a high affinity for LDL and serve to carry the LDL particle into the cell via endocytosis. Once inside the cell, the endocytotic vesicle is broken down by lysosomes and the cholesterol is liberated for use in cellular reactions.

When levels of LDL in plasma are higher than can be efficiently removed by the LDL receptors in the tissues, scavenger cells or macrophages begin to remove cholesterol from these LDL particles. When these macrophages are overloaded with cholesterol they become what are called "foam cells", which are classic components of atherosclerotic plaques.

High density lipoprotein (HDL) is the heaviest of the lipoproteins and it carries about 20% of the blood cholesterol. Studies indicate that this density class is responsible for transporting cholesterol from the peripheral tissues to the liver. In the steady state, tissues excrete cholesterol into the plasma in amounts equal to the amounts taken up from LDL. Such excretion results from cell death as well as membrane turnover in living cells. When cholesterol leaves a cell it is believed to be picked up by HDL. Ultimately the cholesterol in HDL is again returned to the tissues by LDL.

Low levels of HDL cholesterol have been linked to an increased risk in the development of atherosclerosis. In

Figure 2. Illustration of LDL Receptors in Extrahepatic Cells. Cholesterol carrying Low Density Lipoproteins (LDL) bind to high affinity receptors on the cell membrane called coated pits. These pits invaginate into the cell and pinch off to form endocytic vesicles that carry the LDL to lysosomes. In the lysosome, the LDL is exposed to a variety of hydrolytic enzymes that degrade the protein coat of the LDL into amino acids. The cholesterol is then liberated from the lysosomes to met the cells requirements. This delivery of cholesterol results in a decreased intracellular synthesis of cholesterol and the membrane receptors called coated pits. Adapted from Brown (1976).


FREE
CHOLESTEROL
PROTEIN
LDL
BINDING
LYSOSOMAL
HYDROLYSIS
CHOLESTEROL
RELEASE

contrast, high levels of HDL cholesterol seem to protect against heart disease (Miller, 1977).

Based on the functions of each lipoprotein class one must conclude that the ratio of total cholesterol to HDL cholesterol is a more accurate predictor of the risk of coronary heart disease than either HDL or total cholesterol alone.

CHAPTER III

MATERIALS AND METHODS

Experimental Design

Sixty male Nicolas strain large breasted white turkeys, all about two years old, were obtained and placed in eight feet by ten feet pens. There were five birds in each pen. These pens were then randomly assigned to one of four different rations (See Table II for components of rations) and there was a total of fifteen birds receiving one of each of the diets. The birds remained on their respective diets for eight weeks.

Bleeding

Five milliliter blood samples were collected from each bird weekly throughout the test period. A blood sample was also collected before the birds began their respective diet treatments. A vein on the underside of a wing was selected for its accessibility and prominence. Blood samples were drawn from this wing vein using a disposable five milliliter syringe equipped with a twenty guage, one inch needle. Each blood sample was immediately transferred to a screw-capped

TABLE II

Composition of Experimental Diet (percent)

Component	Basal	Basal + Cholesterol	Basal + Dried Eggs	Basal + Whey Protein
Corn	6.26	6.23	4.67	6.26
Soybean meal	24.94	24.80	18.61	24.94
Meat meal	10.00	9.95	7.46	10.00
Whey (protein)	---	---	---	9.06
Cornstarch	25.00	24.88	18.65	20.57
Cerelose	25.00	24.88	18.65	20.57
Animal fat	8.00	8.00	8.00	8.00
Vitamin-TM premix (OSU-100)	.25	.25	.18	.25
Salt	.35	.35	.26	.35
Dical	.20	.20	.15	.20
Cholesterol	---	.50	---	---
Dried Egg*	---	---	25.40	---
Total	100.00	100.00	100.00	100.00

* Dried eggs added to provide equivalent of .5% purified cholesterol

vial containing one milliliter of ten percent ethylene diamine tetraacetic acid (EDTA) pH 7.4, mixed gently by inverting, and stored on ice.

The samples were centrifuged at 2700 rpm for five minutes. Plasma from each sample was transferred to individual small plastic tubes and stored at -28 C until used for analysis. Freezing of plasma samples effects the configuration of lipoproteins. It has been reported however, that rapid freezing and thawing change the lipoprotein structure only slightly (Del Gatto, 1959).

Analysis of Total Cholesterol

Total concentration of cholesterol in the plasma samples was measured by a standard fluorometric method (Albers, 1955; McDougal, 1957). Plasma samples were thawed by placing them in a warm water bath (40 C) for five minutes. After thawing, samples were mixed thoroughly for ten seconds with a vortex mixer and a 20 ul aliquot of plasma was placed in a twenty milliliter test tube. The following sequence of steps was carried out on the 20 ul of plasma:

1. Acetic acid-chloroform reagent was added (0.2 ml). This reagent is a 3:2, v/v ratio of reagent grade glacial acetic acid and chloroform.
2. Chloroform-acetic anhydride reagent was added (5.0 ml). This reagent is a 10:3, v/v ratio of reagent grade

chloroform and acetic anhydride that was mixed fresh before each run.

3. Reagent grade sulfuric acid was added (0.2) and the resulting mixture was agitated vigorously.

4. Samples were centrifuged at 2700 rpm for ten minutes and the clear supernatant was decanted into 12 x 75 mm fluorometer tubes.

5. Forty minutes after addition of the sulfuric acid, fluoresence readings were recorded using a Turner Fluorometer model 111.

Readings were compared to those of standards with known concentrations of cholesterol. Standards were aqueous 100 mg/dl supplied by Sigma Chemical Company. Standards were treated in the same way as samples.

Analysis of HDL Cholesterol

Serum low density and very low density lipoproteins were selectively precipitated by the Magnesium-Phophotungstate method and removed by centrifugation (Lopes-Virella, 1977; Sigma Technical Bulletin No. 350-HDL, 1981). The cholesterol associated with the HDL fraction that remains in the supernatant is then measured using the fluorometric method described above.

The procedure for isolating HDL is as follows:

1. A 0.4 ml aliquot of the plasma sample was placed in a 15 ml conical centrifuge tube.

2. The HDL precipitating reagent, obtained from Sigma Chemical Company, was added (0.5 ml) and stirred with a vortex mixer.

3. The tubes were centrifuged at 2700 rpm for five minutes to obtain a clear supernatant.

4. Aliquots of these supernatants were then analyzed for total cholesterol using the procedure described above.

Statistics

Difference between means was established by standard analysis of variance procedures.

CHAPTER IV

RESULTS

Concentrations of total and HDL cholesterol are presented in figure 3 for day "zero". These values represent normal cholesterol concentrations before the birds in each treatment were subjected to their respective diets. There was no difference in total cholesterol or HDL cholesterol values between any of the treatment groups. The average total cholesterol value for all groups of birds combined was 119.62 $\pm$ 12.08 mg/dl.

Figure 4 shows the average total and HDL cholesterol values for the birds receiving the basal diet. Both total and HDL cholesterol concentrations remained quite constant throughout the trial period. The average total cholesterol concentration was 112.5$\pm$ 11.3 mg/dl. Throughout the trial period no significant change is noticed from the day "zero" value.

Figure 5 represents the total and HDL values for the birds receiving the basal diet plus 9% added whey protein. There was a slight decrease in total cholesterol over the duration of the experiment when compared to the day "zero" total cholesterol value for this group. The average concentration of total cholesterol in the plasma while the

birds were on this ration was 111.0± 8.8 mg/dl. This shows an 7% decrease in plasma total cholesterol from the day "zero" value.

Figure 6 shows the total and HDL values for the birds receiving 0.5% purified cholesterol added to their basal diet. After one week on this experimental diet, total cholesterol levels rose sharply (a 61% increase) from a basal level on day "zero" of 126 mg/dl to 196.8 mg/dl. In these birds plasma total cholesterol increased and reamained high throughout the trial. It is interesting to note that the HDL cholesterol did not increase substantially while the birds were on this diet.

Birds receiving enough dried egg in their diet to supply approximately the same amount of cholesterol as the ones on the purified cholesterol diet showed a 19% rise in plasma total cholesterol levels after one week. However this rise was more gradual and lower in magnitude (a 19% increase) than the elevations of plasma total cholesterol noticed in the cholesterol fed group. These birds exhibited an average total cholesterol value of 142.4± 12.0 mg/dl while on the egg diet. Again, no significant rise in HDL cholesterol was noticed.

Figure 3. Plasma concentrations of total and HDL cholesterol for all four treatment groups at day "zero". These measurements were made before the birds were subjected to their respective diets. Bars represent average concentrations of cholesterol in mg/dl of plasma for fifteen birds in each group. The standard error of the mean is represented by the "T" above each bar. Analysis of Variance test indicates no significant differince (P=.05) between means.

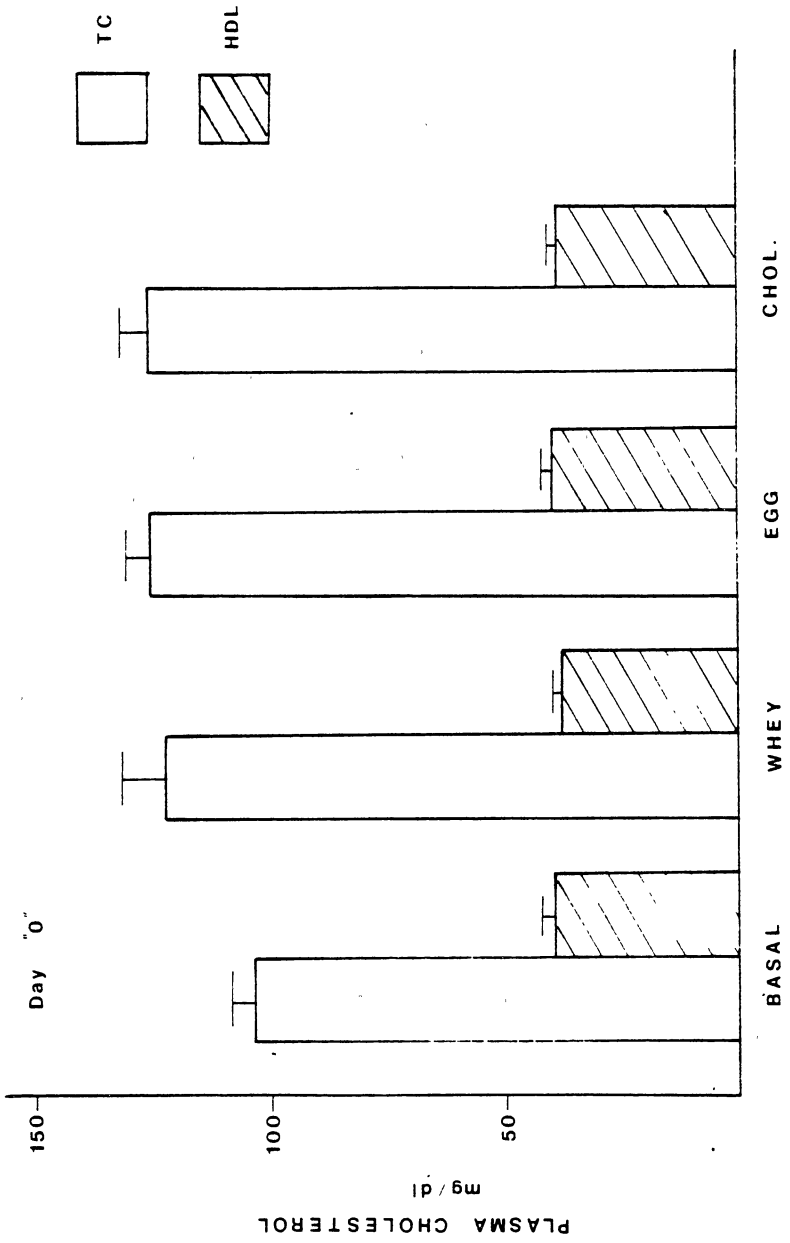

TC
HDL
Day "0"
BASAL
WHEY
EGG
CHOL.
150
100
50
mg / dl
PLASMA CHOLESTEROL

Figure 4. Plasma concentrations of Total and HDL cholesterol for the birds receiving the basal diet. This figure shows the average concentration of cholesterol in mg/dl of plasma. Birds were bled once each week for a period of nine weeks. There is no significant change in plasma cholesterol with time (P=.05)

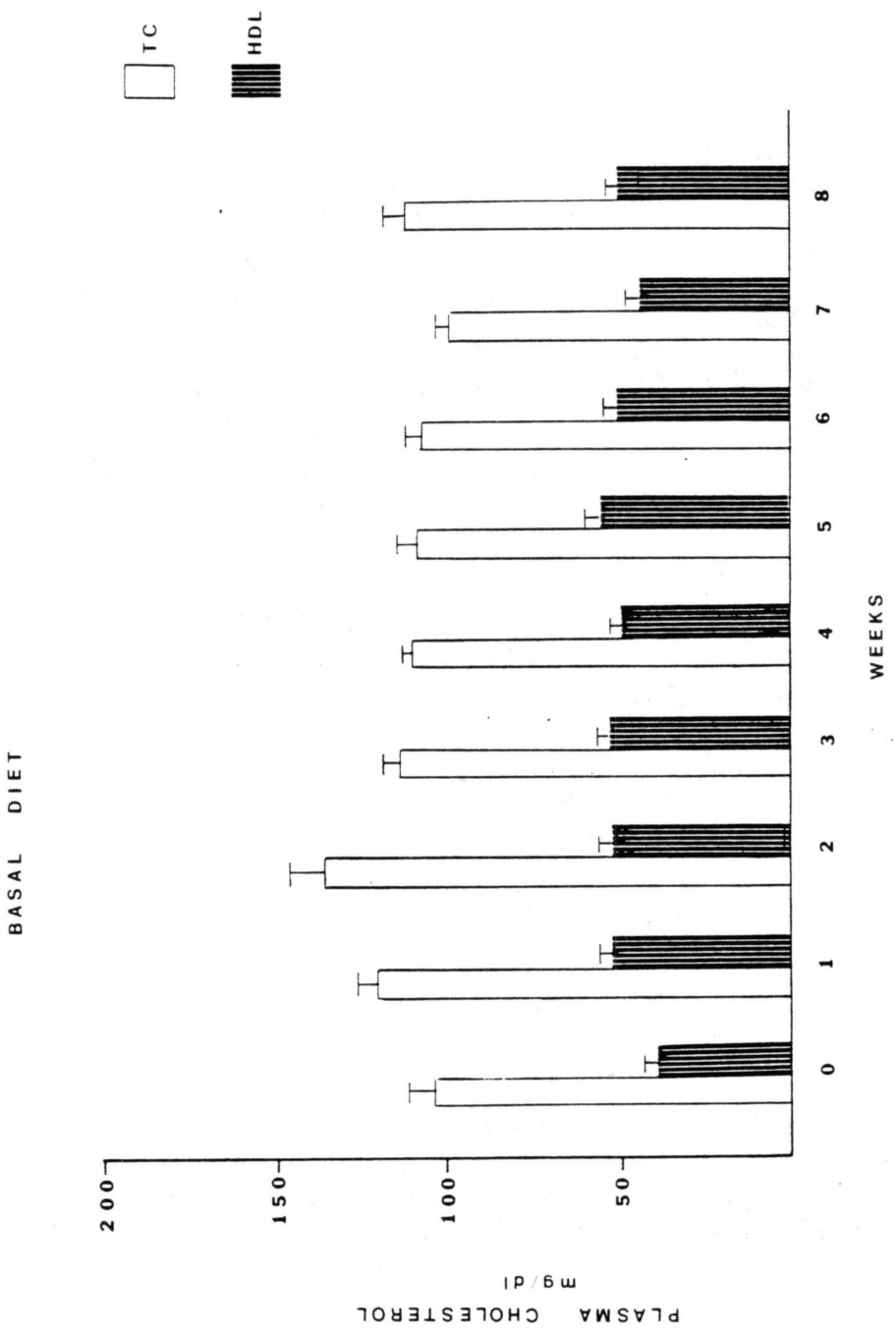

TC
HDL
BASAL DIET
PLASMA CHOLESTEROL
mg/dl
200
150
100
50
0
1
2
3
4
5
6
7
8
WEEKS

Figure 5. Plasma concentrations of total and HDL cholesterol for the birds receiving a diet with 9.0% whey protein. This figure shows the average concentration of cholesterol in mg/dl. Birds were bled once each week for a period of nine weeks. Week zero is the average value before birds were given added whey protein to their diet. Total plasma cholesterol decreased slightly during the eight week period of feeding. The decrease is not significant at the 95% level, but it is significant at the 75% level. The HDL cholesterol values did not change significantly (P=.05) during the eight week period.

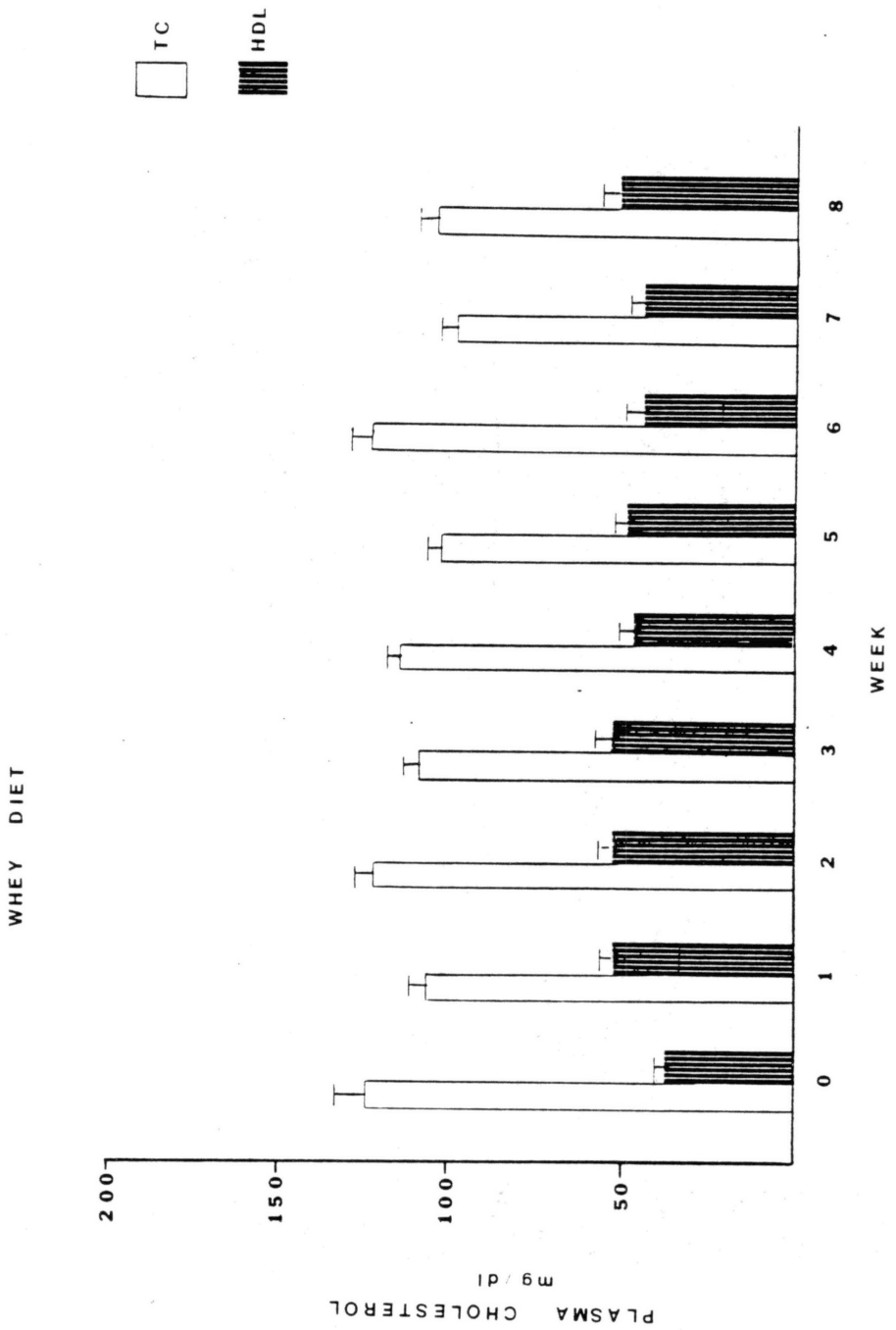

TC
HDL
WHEY DIET
WEEK
0
1
2
3
4
5
6
7
8
PLASMA CHOLESTEROL
mg/dl
200
150
100
50

Figure 6. Plasma concentrations of total and HDL cholesterol for the birds receiving a diet with 0.5% purified cholesterol. This figure shows the average concentration of cholesterol in mg/dl. Birds were bled once each week for a period of nine weeks. Week zero is the average value before birds were given added cholesterol to their diets.Total plasma cholesterol increased significantly (P=.05) during the first week of feeding and remained elevated for the eight week period of the experiment. The HDL cholesterol values did not change significantly during the eight week period.

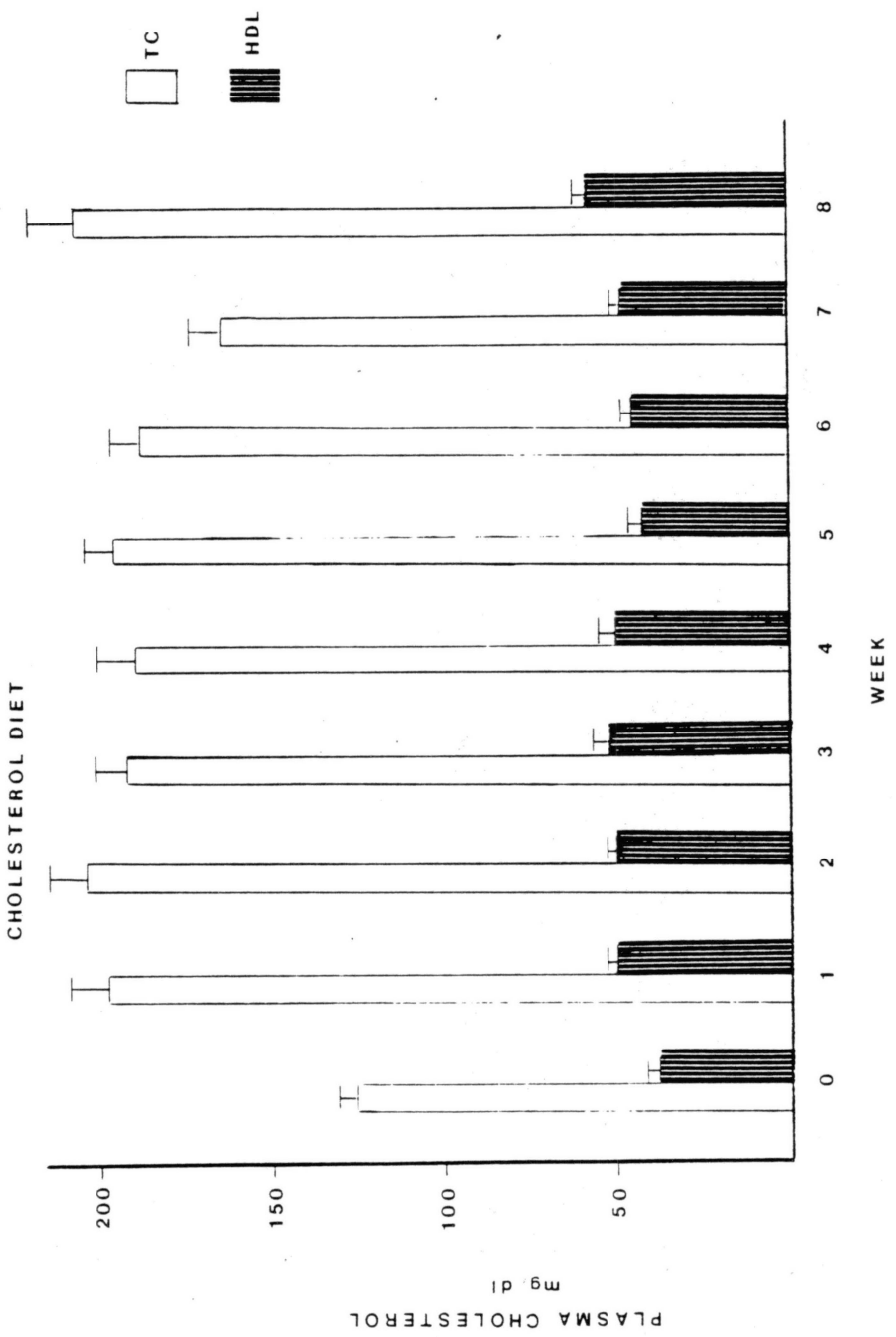
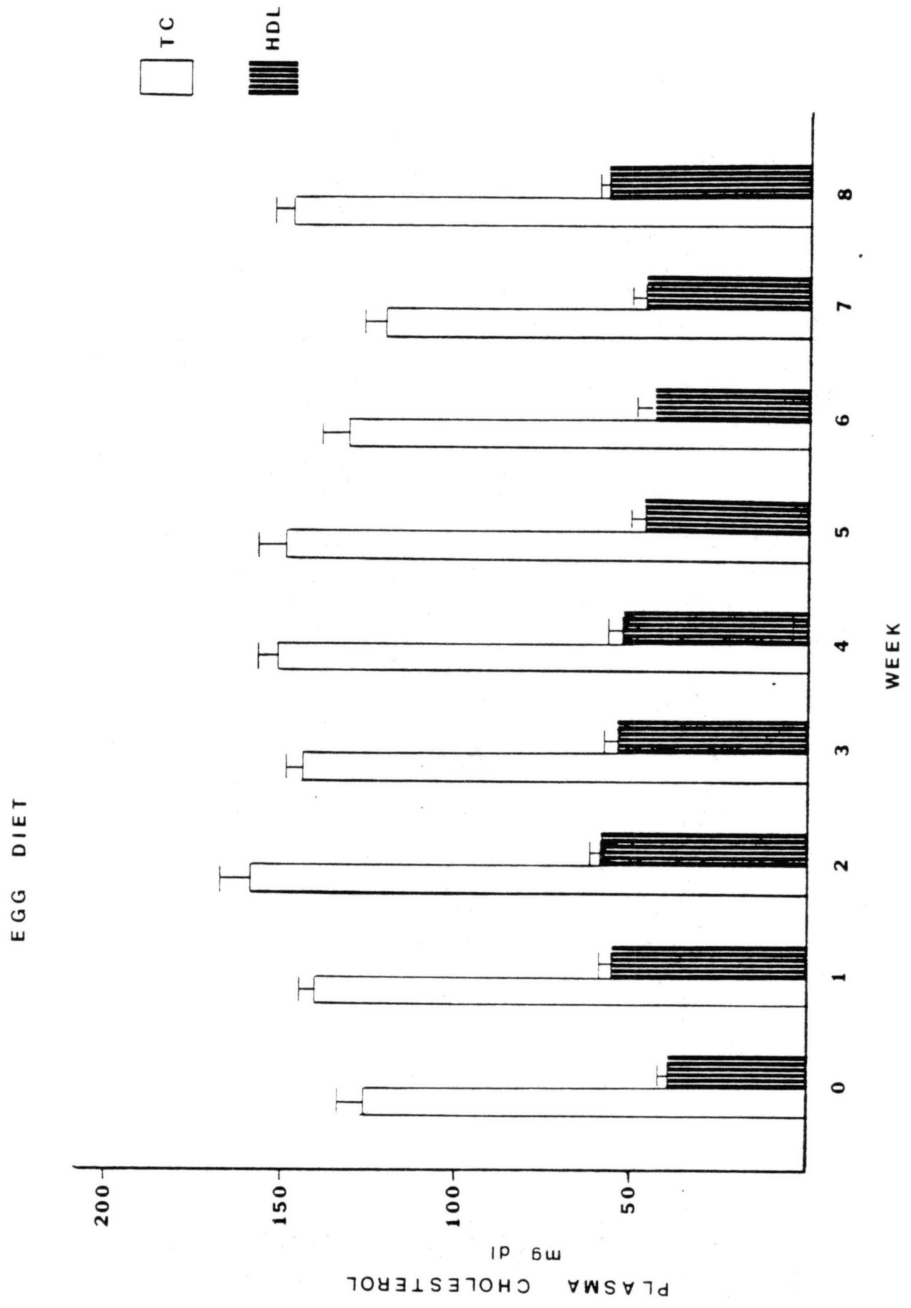

TC
HDL
CHOLESTEROL DIET
WEEK
0
1
2
3
4
5
6
7
8
PLASMA CHOLESTEROL
mg dl
200
150
100
50

Figure 7. Plasma concentrations of total and HDL cholesterol for the birds receiving a diet with enough dried egg added to supply 0.5% cholesterol. This figure shows the average concentration of cholesterol in mg/dl. Birds were bled once each week for a period of nine weeks. Week zero is the average value before birds were given added dried egg to their diet. Total plasma cholesterol increased significantly (P=.05) after two weeks and then declined. The HDL cholesterol values did not change significantly during the eight week period.


TC
HDL
EGG DIET
PLASMA CHOLESTEROL
mg dl
200
150
100
50
0
1
2
3
4
5
6
7
8
WEEK

CHAPTER V

DISCUSSION

This study suggests that whey protein may contain a hypocholesterolemic factor; however, there is some room for doubt. The group of turkeys fed 9.0% whey protein added to their basal diet exhibited a small decrease in total cholesterol (11.0 %) from their basal level. The difference between the whey protein fed group and the group of birds receiving the basal diet was not significant at the 95% confidence level. The means were different at the 75% confidence level (ANOVA). One source of difficulty in comparing birds of different groups was the large variation between individuals in each group.

A remarkable difference in total plasma cholesterol is noticed between birds on the egg diet and birds on the cholesterol diet. Even though these two groups were receiving similar levels of cholesterol in their rations, the birds receiving cholesterol from an egg source had considerably lower plasma total cholesterol than those birds on the diet containing purified cholesterol. This indicates that the inclusion of dried eggs in the diet does not increase plasma cholesterol levels as much as one would assume based on their cholesterol content. It is possible

that the interaction of other compounds in the egg has some effect on the digestion or absorption of cholesterol. Birds in the egg-fed group exhibited a slight downward trend in plasma total cholesterol after the second week on the diet. This was not noticed in the birds on the diet with added purified cholesterol. The gradual decline may indicate that these birds are physiologically adapting to the added dried egg in their diet. Similar gradual declines have been noticed in humans when eggs were added to the diet (Slater, 1976); however, the change was not as marked as the differences observed in this study. Perhaps a longer treatment period would have revealed a new stable value for these birds.

Relatively uniform values for HDL cholesterol among all groups throughout the duration of the experiment indicate that dietary factors probably do not influence the amount of cholesterol carried by HDL. This is in agreement with the hypothesis that HDL levels increase directly with the level of physical activity and not the quantity of cholesterol in one's diet.

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VITA 2

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