THE EFFECT OF DIETARY CALCIUM, CASEIN, AND SOY ON PLASMA CHOLESTEROL IN THE JAPANESE QUAIL (COTURNIX COTURNIX JAPONICA)

By

LORI JOHNSON HAKE

Bachelor of Science

Fort Hays State University

Hays, Kansas

1981

Submitted to the Faculty of the Graduate College
of the Oklahoma State University
in partial fulfillment of the requirements
for the Degree of
MASTER OF SCIENCE
May, 1986

THE EFFECT OF DIETARY CALCIUM, CASEIN, AND SOY ON PLASMA CHOLESTEROL IN THE JAPANESE QUAIL (COTURNIX COTURNIX JAPONICA)

Thesis Approved:

Calvin H. Barnes, Jr.
Thesis Adviser

Mark Maxwell

John A. Brantley

Norman N. Durham
Dean of the Graduate College

ACKNOWLEDGEMENTS

This study demonstrated a hypocholesterolemic effect of dietary calcium in diets containing 0.5% cholesterol. Doubling the daily requirement for calcium reduced the hypercholesterolemic effect of casein to that of the soy diet on diets containing the minimum daily calcium requirement. Dietary manipulation did not significantly effect Plasma HDL. The work was sponsored by Agricultural Experiment Project #1846.

I wish to thank the people who have helped me in this work at Oklahoma State University. I am especially grateful to my major advisor, Dr. Calvin Beames, for his guidance and encouragement. I also wish to thank the other committee members, Dr. Charles Maxwell and Dr. John Bantle, for their advisement. I thank Dr. Robert Teeter for his consultation.

I appreciate Zola Hershey for sharing her knowledge of laboratory procedures. Sharron Norton was extremely helpful in formulating the diets. M. Gene Murray and Deanna Lawson were invaluable in helping to maintain the research animals.

I am extremely grateful for the patience, support, encouragement and constant understanding of my husband, Daniel.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

INTRODUCTION

Incidence of Cardiovascular Diseases

Atherosclerotic cardiovascular diseases are the leading cause of death in the United States (1,2,3,4) accounting for over half of all deaths annually (3,5). This is two to three times greater than the second leading cause of death, cancer (1,3,5). One fourth of all cardiovascular deaths happen to persons under 65 (3). Coronary heart disease (CHD) is insidious, probably beginning in childhood but asymptomatic until midlife (1). The first symptom is often a fatal heart attack or stroke. Half of all CHD deaths occur unexpectedly; seventy percent occur outside the hospital (3).

Cardiovascular Risk Factors

The prevalence of atherosclerotic disease has lead to intensive research into its multifaceted etiology. Exact mechanisms have not yet been elucidated but risk factors have been identified. Primary risk factors include genetic predisposition, blood lipid levels, hypertension and smoking (1,2,3,5). Identification of preventative measures are needed.

A large body of epidemiological, clinical and experimental evidence point to a positive correlation between total serum cholesterol, low density

lipoproteins (LDL) and atherosclerosis. It is uncertain whether the relationship between high blood cholesterol and CHD is causal or associative. However, growing consensus is that total serum cholesterol level is a reliable indicator of latent risk of CHD and that the magnitude of the problem is too great to await definitive proof (1-7).

A strong negative correlation exists between plasma high density lipoprotein (HDL), CHD mortality (3) and lesion growth (8). The total cholesterol:HDL cholesterol ratio is considered the best predictor of CHD risk within a population, especially for people over age 55 (3).

Optimal Total Cholesterol Levels

Optimal levels of total serum cholesterol have been established which correspond to decreased risks of CHD mortality in humans (1,2,3,5,7). Blood cholesterol levels above 200 - 230 mg/dl are considered at increased risk of premature CHD (1,2,5). Fifty percent of the United States population falls in this risk category. The risk level has been further divided into moderate and high risk. The National Institutes of Health recommend dietary intervention to lower blood cholesterol levels, reserving pharmacological treatment for those at highest risk (1).

Diet and Atherosclerosis

The thirty-one percent decline in cardiovascular (CV) disease mortality from 1970 to 1980 has coincided with lifestyle and dietary changes and lowering of serum cholesterol values (3,8). In 1976 almost half of all Americans had

altered their eating patterns for health concerns (8).

Dietary factors play a significant role in levels of blood lipids. Extensive research into effects of specific dietary factors on serum lipids seek to determine individual effects, as well as interactions of dietary components, on the absorption, transport and metabolism of lipids (5).

Dietary cholesterol, fat and animal proteins correlate positively with CHD mortality (9). Animal proteins have been demonstrated to be more cholesterolemic than vegetable proteins. Saturated fats elevate blood cholesterol more than unsaturated fats (10, 11). The role of many minerals has yet to be determined.

Current dietary recommendations deemphasize dairy products (1,3,5). Concern exists that such changes to modify cholesterol consumption may be harmful (12) and perhaps decrease intake of essential nutrients such as calcium, although such a trend has not been confirmed (13).

Is Calcium Hypocholesterolemic?

Epidemiologic studies have negatively correlated CV death rates with water hardness (14), particularly with water calcium levels (15, 16, 17, 18). Animal and human diet studies indicate calcium may be hypocholesterolemic (19,20,21) and antihypertensive (22,23,24).

Intent of Study

The intent of this study is twofold. First, the effect of two levels of dietary calcium on plasma cholesterol and HDL will be examined. Second, the

cholesterolemic effect of two protein sources, animal (casein) and vegetable (soy) will be compared. Japanese quail (Coturnix coturnix japonica) will be used as the experimental model.

CHAPTER II

LITERATURE REVIEW

Dietary Calcium

Physiological Role of Calcium

Calcium is the most abundant essential mineral in the body. Ninety-nine percent is in the skeleton and teeth. The remaining one percent, in the body fluids and cells, is involved in regulation of important functions including normal muscle and nerve function, maintenance of the intracellular environment and hormonal action (25,26). Calcium is a "second messenger" mediating cellular responses to a variety of stimuli. Plasma calcium levels are narrowly maintained in man at 9-11 mg/dl ± 3%. Vitamin D, parathyroid hormone, calcitonin and other hormones regulate plasma calcium levels (27,28).

Despite the crucial role of calcium, controversy exists on the required daily intake of dietary calcium (27,28). Total body calcium is regulated primarily by the amount of dietary calcium absorbed (28). The United States Recommended Dietary Allowance (RDA) is 800 mg daily, twice the FAO/WHO Committee on Calcium Requirements recommended 400-500 mg daily. The RDA is considered too low, especially for the elderly, by many (28) to maintain calcium balance and in excess by others (27).

Different diets and physiological states effect calcium bioavailability. The efficiency of calcium absorption increases when intakes of calcium are low (27, 28,29). It has been assumed that the rate of calcium absorption will "adapt" to low calcium intakes so that dietary intake is not crucial. Recent observations suggest, however, that such adaption is not sufficient to maintain calcium balance and optimal bone mineralization (28).

Intestinal Absorption and Transport of Calcium

Evidence suggests that calcium levels are well regulated at the level of absorption (26). Only a portion of dietary calcium is absorbed. The fraction that is absorbed depends on the presence of other dietary components, the calcium and vitamin D, and the physiological state of the individual (i.e., growth or pregnancy) (28). Urinary excretion of calcium, in man, occurs when blood calcium exceeds 7 mg/dl and since plasma calcium is regulated betweehn 9 - 11 mg/dl urinary calcium excretion remains relatively constant. Fecal excretion of calcium varies in response to diet (26) and this variation is assumed to reflect changes in calcium absorption (28).

Dietary calcium is usually complexed with other dietary constituents. The calcium must be released in a soluble, and probably ionized form to be absorbed (28). Fecal calcium tends to increase in high protein diets (26). The formation of insoluble calcium soaps (with fatty acids) or inorganic calcium salts (with oxalates, phytates and phosphates) inhibit calcium absorption (26).

Gastric acid seems to increase solubility of calcium complexes (28). Calcium precipitates from solution with a pH over 6.1. Calcium binding proteins

(CaBP) in the intestinal mucosa bind and play a role in absorption of calcium. Synthesis of these proteins are induced by vitamin D in low calcium diets (26,29). Calcium binding proteins are found mainly in the duodenum and upper jejunum where calcium absorption primarily occurs (28).

Solubility of calcium salts in vitro and intestinal absorption of calcium is increased by bile. Bile salts are needed for optimal vitamin D absorption. In man, calcium salts present in bile acids seldom precipitate and bile calcium may be preferentially absorbed. Bile is a route of secretion of calcium into the intestine (28).

Calcium absorption is not completely understood but appears to happen by two mechanisms: a nonsaturatable, simple passive diffusion and a saturatable transepithelial carrier mediated process. The latter is most significant when the interluminal calcium concentration is low. Diffusion is most important when large calcium concentration gradients exist (29). The carrier mediated transport probably accounts for increased calcium absorption with low dietary intakes or when physiological demand for calcium is high (25,29).

Dietary Calcium and Cardiovascular Disease

The "Water Story". Epidemiological evidence suggests some minerals in water may effect the development of cardiovascular disease, especially hypertension and atherosclerosis. Studies have found cardiovascular death rates inversely correlated with drinking water hardness (15,16,17,30). Schroeder (31) found the range of cardiovascular deaths to be nearly forty-three percent less annually in New Mexico, a hard water state, than South Carolina, a

soft water state. The protective or enhancing substance in water has not been identified (30) but may be due to one of the principle cations (calcium or magnesium) that contributes to water hardness (17).

A study in England and Wales found cardiovascular mortality altered in boroughs where the water hardness had changed substantially over a thirty year period. Cardiovascular death rates decreased in towns where the water had become harder and increased where it had become softer; noncardiovascular mortality did not correlate with water hardness changes (31). Another English-Welsh study found highly negative correlations between water hardness and CV mortality which were even more negatively correlated with water calcium (31).

In areas where the water is "hard", drinking water accounts for seven percent (17) to fifteen percent (32) of man's total mineral intake of calcium and twelve percent (17) for magnesium. The magnesium value is probably overestimated due to lack of nutrient data for magnesium in food (17).

A postulated mechanism for the protective effect of calcium or magnesium is that these minerals may protect against the uptake of potentially toxic metals (Mn and Pb) from the gut. Calcium and lead may compete for CaBP. It is not known whether increased uptake of Mn and Pb is a risk factor for CV disease (16).

The Hypocholesterolemic Factor(s) In Milk

Dairy products are high in saturated fats and cholesterol, which are hypercholesterolemic. Evidence suggests, however, that milk products may

contain a hypocholesterolemic factor. Possible hypocholesterolemic factors include: hydroxymethyl glutarate, orotic acid, lactose and calcium (33,34).

Mann (35) found low CVD and serum cholesterol in the Masaii who have a high intake of milk and meat. Thakur and Jha (33) found yoghurt, milk and calcium supplemented diets to reduce dietary cholesterol induced hypercholesterolemia in rabbits. Yoghurt and calcium diets were more hypocholesterolemic than milk diets. Milk and yoghurt consumed in this study had equivalent calcium. This suggests calcium is hypocholesteroemic but that other factors may be present.

Hepner (36) reports the hypocholesterolemic effect of yoghurt may be due to its significant calcium content, supplying 864 mg/day in his study. The hypocholesterolemic factor in yoghurt is not due to the pasteurization or non-pasteurization of yoghurt (36).

Studies of male prisoners found no significant change in serum cholesterol, triglycerides of high density lipoproteins in control, skim milk, or buttermilk diets (34). Rat diet studies have found milk not to be hypolipidemic or hyperlipidemic. Compared to the control diet, however, the milk diet did not raise lipid levels despite ingestion of fifty percent more fat, nine milligrams of cholesterol a day and thirty-five percent less fiber (37).

Rossouw et al. (38) varied human diets with skim milk, yoghurt and full cream. Serum cholesterol was lower with skim milk and increased at two weeks with yoghurt and full cream. The changes correlate with dietary fat and cholesterol intakes. No convincing evidence of a hypocholesterolemic milk factor was seen. The high density lipoprotein (HDL) particles are considered

protective against atherosclerosis; they increased with each of the three diet treatments.

A metabolic ward study featuring a high calcium control (2600 mg/day), a low calcium control (800 mg/day) and a high calcium skim milk diet (2600 mg/day) indicated no hypocholesterolemic factor in milk (39). On the high calcium control the plasma cholesterol:HDL ratio decreased and the triglycerides in LDL increased. On high calcium skim milk the plasma cholesterol:HDL ratio decreased. HDL decreased, plasma cholesterol and LDL increased. The plasma cholesterol in the skim milk diet was about ten percent higher in seventy-eight percent of the subjects than in the high calcium control, but this difference was not statistically significant.

Cottage cheese is not hypocholesterolemic suggesting the hypocholesterolemic factor is in whey. Whey consists of whey protein, lactose, vitamins and mineral salts, including calcium. Howard and Marks (40) found only butter to be hypercholesterolemic. There was a lack of effect of lactose and lactose plus calcium and magnesium suggesting that the cholesterol lowering factor is not lactose or lactose in combination with mineral salts (40).

Lactose increases cholesterol absorption while calcium has been known to decrease cholesterol absorption in lactose containing diets. Wells and Cooper (41) saw two percent calcium chloride in a lactose diet lowered total liver cholesterol and ended mild diarrhea in lactose fed rats. When added to fat-free diets calcium chloride had no effect on cholesterol absorption or lowering of liver cholesterol levels.

The hypocholesterolemic factor in milk and yoghurt may be calcium or it may be calcium in association with other, as yet unknown factors.

Dietary Calcium and Serum Lipid Levels

Animal and human experiments indicate that dietary calcium is hypolipidemic. Yacowitz, et al. (42) fed Holtzman rats corn oil or saturated fat at three calcium levels (.08, .2, and 1.2%) and found dietary calcium to be hypocholesterolemic and hypotriglyceridemic for both fats, but with greater effect in saturated fat diets (42). At the 1.2% calcium level in cocoa butter diets, serum total lipids were reduced to levels comparable to those on the corn oil diet. Addition of 1.2% calcium lowered cholesterol and triglycerides with both fats.

Fleishman et al. (43) fed Holtzman rats a corn-soy ration with 15.63% protein, beef tallow and 2% added cholesterol. Calcium was added as calcium carbonate at 0.08, 0.2 and 1.2% levels. Blood lipids decreased with increasing calcium. The major decline occurred at 0.2% calcium with a small, additional decrease in total cholesterol and serum lipids at 1.2%. In another study Fleischman, et al. (44) fed Holtzman rats diets with 2% calcium carbonate, calcium carbonate plus D_2 and oyster shell calcium plus D_2. Serum cholesterol levels were lower than control by 35%, 27%, and 22% respectively. Triglycerides were lowered by 10%, 10% and 17%. The hypotriglyceridemic effect of oyster shell calcium may be due to the increased magnesium and zinc content of oyster shell calcium; both are known to be hypolipemic. Serum phospholipids decreased in calcium carbonate supplemented diets, but remained the same in the oyster shell group. Serum free fatty acids (FFA) were

32% higher in the calcium carbonate diets and 55% higher in the oyster shell group.

Goats fed a calcium supplement had a 16.6% decrease in plasma cholesterol from the basal milk diet level (45). Rabbits fed a low fat diet at 0.02, 0.8 and 1.6% calcium resulted in elevated plasma cholesterol and phospholipids at the 0.02% level with no significant decrease at higher calcium supplements. Triglycerides showed no change. The lack of lipid lowering effect may be due to the low fat content of the diet (20).

Infusions of calcium carbonate by stomach tube at 2 - 4 grams daily prevented an increase in serum cholesterol and phospholipid, but not triglycerides, resulting from acute starvation. Not surprising, a decrease in serum calcium levels with starvation was also prevented (46).

Human studies have also provided conflicting results on hypocholesterolemic effects of calcium. Yacowitz (47) supplemented four men with 2.66 grams per day of calcium. A decrease in serum cholesterol was observed in every case. Bieranbaum (19) gave two grams of calcium carbonate each day to eight hyperlipemic men and two women. A 25% decrease in serum cholesterol levels occurred over a year. A reversal of the cholesterol to phospholipid ratio from 1.04 to 0.92 was observed. Serum triglyceride and phospholipid decreased but not significantly. No side effects were seen.

A 750 mg calcium plus 375 mg D_2 supplement of elderly women resulted in a striking, highly significant decrease in serum cholesterol (21). A "weak" hypocholesterolemic effect was seen with two gram calcium supplementation of sixteen hyperlipidemic patients. Serum triglycerides did not change (48).

No change in serum cholesterol of triglyceride was seen in six patients (five were osteoporotic) given either organic salts (calcium glycerophosphate or calcium glucono-galactogluconate) or skimmed milk. A definite effect on cholesterol metabolism was indicated however (49).

In eleven men supplemented at two calcium levels in saturated fat diets a small lowering of borderline statistical significance of serum cholesterol occurred. No change in serum cholesterol occurred n polyunsaturated fat diets with calcium supplementation (50).

Dietary Calcium and Tissue Lipids

Animal studies show a relationship between levels of dietary calcium and tissue lipids. Fleischman et al. (43) sacrificed Holtzman rats fed a high saturated fat diet with 2% added cholesterol with varying calcium levels. His results indicate that the decrease in serum lipids with increasing calcium intakes was not caused by deposition of those lipids in the tissues examined. No increase in tissue lipids corresponding to a decrease in serum lipids was seen. Liver cholesterol did drop 17% at the 0.2% calcium level with no more decrease at 1.2% calcium level. No changes in heart lipids or liver total lipids or phospholipids was observed.

Yacowitz et al. (42) Holtzman rats fed corn oil or saturated fat diets at three calcium levels did not show a change in liver total lipids of cholesterol with increasing calcium. At 0.08% calcium, the corn oil diet rats had a higher deposition of lipids in liver and triglycerides in lungs compared to cocoa butter

fed rats. Increased calcium corresponded with lower triglycerides in lungs with rats fed unsaturated fats.

Iacono (20) fed rabbits a low fat diet with three calcium levels. On the calcium deficient (0.02%) diet, liver cholesterol and phospholipids were high; lung cholesterol and phospholipids were low. On the high calcium diet heart, adrenal gland and skeletal muscle had a decrease in free cholesterol. Total phospholipids decreased in skeletal muscles. Tissue triglycerides increased in the kidney, skeletal muscle, and adrenal gland with increasing dietary calcium. Triglycerides varied independently of cholesterol and phospholipids .

In a study with goats, Hines et al. (45) found the total lipid and cholesterol concentrations unaltered in liver, other viscera and carcass tissues. Increasing dietary calcium correlated with decreased total lipids in aortas. Addition of dietary cholecalciferol (D_3) increased cholesterol content of the aortas. Rabbits fed increasing levels of calcium (.08, 0.2, and 2.0%) had decreasing aorta cholesterol content and decreased severity of plaque scoring (1.9+, 1.4+, and 1.9+ respectively) (51).

Dietary Calcium and Dietary Fats

Dietary fats, which are usually trigycerides of long chain fatty acids, are emulsified in the intestinal lumen. Pancreatic lipase hydrolyzes ester linkages giving insoluble monoglyceride (MG) and free fatty acids (FFA) (52). The lipolysis products are rendered soluble by complexing with bile salts, which have detergent properties, to form particles called micelles. As micelles, the MG and FFA's are exposed to the apical surface of the intestinal cells where they are

absorbed. Bile salts are not absorbed at this point and can be and are reused. In bile, mixed micelles prevent precipitation of free cholesterol and phospholipids and subsequent gallstone formation by solubilizing the steroids with bile salts (53). Bile is an obligatory requirement for passage of cholesterol from intestinal lumen and lymph (54).

In the upper intestine either calcium or magnesium can form insoluble soaps which are in turn poorly absorbed (52). In 1918 Bosworth (55) noted infants ingesting cow's milk, richer in soluble calcium salts than human milk, had increased calcium soaps in their stools. Fecal fat (52) and fecal calcium (56) excretion increases significantly with increasing amounts of dietary calcium. The extent of soap formation with fats depends on ionized calcium levels in the intestinal lumen, though the solubility of soaps formed determines actual amounts appearing in feces (48).

Excess dietary calcium seems to interfere with absorption of fat in poultry and rats (46,54). Increasing any type of dietary fat above five percent decreases calcium, iron, magnesium and zinc retention in chicks (58).

The effect of dietary calcium on fat absorption varies with the type of fat. Removal of calcium (or magnesium) from a diet dramatically increased the digestability of fats. For example, the digestion of trilaurin is increased by 17% by the removal of calcium. A decrease in fat excreted as soap and an increase in the neutral free fatty acid fraction was also observed. Effects of calcium and magnesium salts are progressive, as their amount increases so does the observed effect (56).

In lambs, decreased ration digestibility by corn oil was alleviated by calcium carbonate and/or calcium chloride. Magnesium was not effective. It is thought that fatty acids are acting as antimetabolites to rumen bacteria, thus decreasing ration digestability. The addition of calcium causes precipitates of the fatty acids as calcium soaps and allows the remainder of the ingesta to ferment normally. Fecal calcium increased with added dietary calcium (59).

Saturated fats are hypercholesterolemic. Lauric, myristic and palmitic acids are twice as effective in raising cholesterol as polyunsaturated fats are at lowering them. A high polyunsaturated to saturated fat ratio is recommended in cholesterol lowering diets (60).

Polyunsaturated fats decrease blood cholesterol and intestinal cholesterolgenesis and sometimes increase liver cholesterol concentration (61,62). The hypocholesterolemic mechanism of polyunsaturated fats may be due to decreased cholesterolgenesis in the intestine, or redistribution of cholesterol from plasma to liver (61) but does not involve inhibition of intestinal absorption of sterols (62). If bile cholesterol excretion is an index of hepatic turnover of cholesterol, polyunsaturated fats may reduce plasma cholesterol by increasing cholesterol turnover since polyunsaturated fats increase production of bile acids in animal experiments (62).

Increased ingestion of calcium increases fecal cholesterol and degree of saturation of fecal lipids in rats (43). Saturated fat diets supplemented at 3 increasing levels of calcium resulted in 44, 52, and 103% greater fat excretion than polyunsaturated fat diets supplemented at 3 calcium levels. The hypo-cholesterolemic effect of calcium is more pronounced in saturated fat diets (42).

Dietary Calcium and Serum Calcium Levels

Dietary calcium level has no effect on serum calcium levels in women (21) and rabbits (46). In calcium supplemented women, serum calcium, serum phosphorus and their ratios, and urinary calcium excretion shows no change (21). Holtzman rats in calcium fed diets had increased serum calcium with no change in total calcium, magnesium, calcium:magnesium ratio, inorganic phosphate, proteins or albumins in their serum (33).

The Role of Calcium in Arterial Calcification

An adequate calcium supply is necessary for atherogenesis to occur (45). Atheromatous lesions are characterized by lipid accumulation, blood products, fibrous tissue and large deposits of calcium and magnesium. It is unknown whether calcification is a primary or secondary condition in atherosclerosis (27). Once extensively calcified the lesions do not regress (45).

Moon (27) asserts that United States calcium intakes are too high and that this alone is sufficient to cause arterial calcification. Studies in goats do not support Moon's theory. Aortic calcification in goats was increased five fold when dietary calcium and D_3 were fed together in large quantities but not when dietary calcium or D_3 were given alone. Aortic magnesium deposits were higher in calcium plus D_3 fed animals (45). Dietary cholecalciferol (D_3) is a positive risk factor for atherosclerosis. Hypervitaminosis D is atherogenic (27,44,45).

Goats fed differing calcium levels and/or D_3 showed no change in serum calcium concentration. Aortic lipids were lowered in D_3 supplemented groups when calcium supplementation was added; dietary calcium lowered aortic lipids even when very high levels of D_3 were fed (45). Increased dietary calcium has been shown to reduce atheroma formation (51).

Calcium depositions may not be directly caused by high levels of calcium but by the action of vitamin D on arterial cells (45). One theory proposes that hypercholesterolemia, in addition to developing lipid complexes in lesions, may function as an agent sensitizing arterial cells to calcium ("calciphylactic hypersensitivity"), resulting in increased calcium deposition (27).

High dietary or serum cholesterol could result in an increase synthesis of vitamin D_3. In the intestinal wall dietary cholesterol converts to a 15:1 cholesterol: 7-dehydrocholesterol (provitamin D_3) equilibrium mixture (27). Hines, et al. (45) suggests that the main effect of dietary calcium and D_3 may be their influence on aortic calcification rather than cholesterol and total lipid metabolism (45).

Dietary Calcium and Hypertension

The National Center for Health Statistics, Health and Nutrition Examination Survey I (HANES I) found low calcium consumption was the most consistent factor in hypertensive persons. Dietary intake of calcium, potassium and sodium were positively correlated with lower mean systolic blood pressure and risk of

hypertension (22). Serum calcium was directly related to diastolic pressure in white people (24).

Calcium supplementation of young adults resulted in a significant decrease of 5.6% and 9% in the diastolic blood pressure of women and men, respectively. No difference in serum calcium was found (35). Low serum concentration of ionized calcium has been found in humans with hypertension (64).

A lack of effect of oral calcium supplements on blood pressure and vascular response was seen in normotensive men. Blood pressure, serum calcium, ionized serum calcium, serum magnesium, and urinary calcium were not altered with dietary calcium supplementation (65).

The mechanisms involved in the relationship between calcium and hypertension are unclear (64), but they may be due to calcium's vasorelaxing, membrane stabilizing effect in vascular smooth muscle which could decrease vascular resistance (23). In the HANES I survey dietary calcium intake was inversely correlated with body mass and this may be related to decreased hypertension observed (22).

The Atherogenicity of Dietary Proteins

The relationship between animal and vegetable proteins and serum cholesterol levels was first recognized by Ignatoswki in 1909 (66). Subsequent research, however, focused on the cholesterol content of animal and vegetable diets rather than on the proteins themselves. In 1941 Meeker and Kerter found casein to be more atherogenic in the absence of dietary cholesterol than soy

protein diets in rabbits. Casein plus 60 mg of cholesterol induced atherosclerosis six times as severe as a similar soy protein diet (67). Experimentally, casein is often used as a representative animal protein; soy is the usual vegetable protein used (10).

In rabbits, casein affects cholesterol absorption , excretion, and transport more than soy protein (10). Carroll (68) found casein hypercholesterolemic and atherogenic but not soy. Casein fed rabbits had a slower plasma cholesterol turnover, less excretion of neutral steroids and bile acids and greater cholesterol absorption . Excess cholesterol in casein fed animals is reflected by increased low density lipoprotein (LDL) 68,69). In mice, soy diets decreased plasma cholesterol and increased intestinal bile acids, suggesting increased bile secretion may mediate the hypocholesterolemic effect (70). In rats, casein results in higher serum cholesterol than soy diets with a corresponding increase in the VLDL fraction. Kinetic studies indicate that the accumulation of VLDL in rats fed casein diets is due to deficient VLDL removal by the liver (71).

A casein diet which increases serum cholesterol, LDL and VLDL suggests a decreased clearance of plasma cholesterol - perhaps due to a decreased number of LDL receptors in the liver, which in turn is expressed by a reduced biliary steroid output (72).

In chickens fed soy, egg white or lactalbumin protein diets, only a very small decrease in plasma cholesterol occurred in the soy diet (73). A study of protein quality and quantity in chickens found no difference in soy or casein feeding on serum or liver cholesterol. Twenty percent protein diets, either soy or

casein, increased cholesterol and VLDL. Fifty percent soy or protein diets prevented the hypercholesterolemic response (74).

Experiments involving the feeding of different levels of soy or whey to Japanese quail gave results which indicated that plant and animal proteins were both hypocholesterolemic at high levels (75).

Various human studies have found no change in serum cholesterol with soy or protein diets (76,77,78,79) although an increase in HDL was observed on isolate soy diets (76,78). One study of people fed animal or vegetable protein revealed a population of responders, whose serum cholesterol were lowered by experimental diets, and a portion of nonresponders, who showed no change. In the responders both soy and casein were hypocholesterolemic. Animal protein diets were more hypocholesterolemic (16% reduction) than soy protein (13% reduction) (80). In monkeys no change in serum total cholesterol with different types of protein diets was seen, although HDL increased and VLDL decreased in soy protein diets (81).

A study feeding three proteins (caseinate, soya isolate, and soya concentrate) to three species (man, rabbit and rat) found caseinate to increase total cholesterol in rabbits, slightly in rats and none at all in humans. This indicates the problems in extrapolating results from animal studies to humans (78). It is uncertain whether casein is hypercholesterolemic or soy is hypocholesterolemic. Mechanisms of action have not been determined.

The amino acid composition, specifically the lysine/arginine ratio, may account for the differing cholesterolemic effects of these proteins. The lysine/arginine ratio in casein is 2; for soy is 1. Adding lysine to soy diets

increases cholesterol turnover time in rabbits and expands body cholesterol pools. Casein with additional arginine is not less hypercholesterolemic than casein alone. Diluting casein with soy normalizes cholesterolemic effects of casein as the ratio approaches 1:1 (82).

Van der Meer proposed that the hypercholesterolemic effect of casein is related to its phosphorylation state. Calcium may bind to casein, a highly phosphorylated protein, forming calcium phosphate sediments which reduces calcium availability for interaction with biliary micelles. More micelles would be available for reabsorption, thus decreasing steroid excretion and increasing cholesterol. Additional dietary calcium could ameliorate the cholesterolemic effect of casein (83).

Samman and Roberts extended this hypothesis to include casein interfering with the bioavailability of other divalent cations, zinc and copper, in the intestinal lumen. Their data in rabbits suggests supplementation of zinc or copper may render casein less hypercholesterolemic (67).

Cholesterol

Description

Cholesterol (3-hydroxy-5,6-cholestene) is a steroid usually found in association with animal fats. It is synthetized in animal tissue and is essential for normal cell function. It is a precursor to all steroids synthesized in vivo. Endogenous sythesis contributes to most of the body cholesterol; dietary sources provide a small fraction. Elimination occurs by conversion to bile acids

or as excretion of fecal neutral steroids. High plasma cholesterol is positively correlated with atherosclerosis and accumulates in atherosclerotic plaques (84,85,86).

Absorption of Cholesterol

Endogenous (biliary) and exogenous (dietary) cholesterol are present in the intestine. The intestinal mucosa also synthesizes small amounts of cholesterol. Unesterified biliary cholesterol outputs are 800-1200 mg daily compared to 400-500 mg daily of dietary cholesterol in adults (84).

Dietary cholesterol is deesterified by pancreatic cholesterol esterase (84). Bile acids, fatty acids, monoglycerides and lysolecithin form mixed micelles with cholesterol and are essential for cholesterol absorption (53,54,84,85). Cholesterol enters intestinal cells by monomolecular passive diffusion through the lipid of the membrane. The rate limiting step is absorption is probably not penetration but passage through the unstirred layer (84).

Cholesterol Transport

Once absorbed, hydrophobic cholesterol and other lipids are transported in the blood and lymph as lipoproteins. The largest lipoproteins, chylomicrons and very low density lipoproteins (VLDL) are droplets of triglyceride and unesterified cholesterol emulsified by a surface layer of phospholipid and free cholesterol (53).

VLDL are derived from the intestine and liver. They carry 2% protein and 98% lipid. Eighty-seven percent of these lipids are triacylglycerol, 8%

phospholipid, 3% cholesterol ester and 1% free cholesterol. VLDL carry lipids from the liver and gut to peripheral tissues (86).

Chylomicrons release FFA from TG after hydrolysis by lipoprotein lipase, leaving chylomicron remnants (84). In the liver, cholesterol esters of chylomicrons are hydrolyzed and taken up by the liver. A portion of VLDL are formed in the liver and transport cholesterol in the plasma (86). VLDL encounter lipoprotein lipase in capillaries in peripheral tissues releasing fatty acids which enter cells of adipose and muscle tissue (87). This leaves intermediate density lipoproteins which convert to low density lipoproteins (LDL) by acquiring more cholesterol ester in their core.

LDL may also be secreted by the liver, independently of VLDL by an unknown method (84). LDL particles are TG poor and contain 70% of the circulating cholesterol in man. Cholesterol may enter vessel walls as LDL (87). Eighty percent of LDL is removed from the plasma by specific cell surface receptors which promote internalization and release of cholesterol in LDL (88). Polar lipids and apolipoproteins left from the LDL transfer to HDL. HDL particles carry cholesterol away from tissues to the liver, where HDL is a precursor for bile acids and biliary cholesterol, thus providing a route of cholesterol elimination from the body (97).

Nascent HDL discs are secreted by the liver and pick up cholesterol from cells. Lecithin cholesterol acyl tranferase enzyme esterifies free cholesterol to cholesterol esters which are hydrophobic. These enter the core of HDL, making it spherical (88). A fraction of HDL, HDL-I, may reduce cellular uptake of circulating cholesterol by competing with LDL for lipoprotein receptors on

smooth muscle cells (87). In vitro, HDL can decrease uptake of LDL by aortic smooth muscle, vascular endothelium and fibroblasts. HDL may be protective against atherosclerosis because of these functions (88).

The Japanese Quail as an Experimental Model in Atherosclerosis Research

The Japanese quail (Coturnix coturnix japonica) has proved a useful animal model for atherosclerosis research (89,90,91) particularly in nutritional (92,93,94) and pharmacological (90) studies.

Quail are small, omnivorous, easily handled and cost effective (92,95). Sexual maturity is reached at 42 days from hatching. Physiological aging is fast and lifespan short (95).

Unlike rats and mice (90,91) quail are susceptible to spontaneous or cholesterol induced atherosclerosis (92). Birds fed added cholesterol at 0.5%, as in this study, developed atherosclerosis, where quail not fed cholesterol did not develop it (90).

Quail, especially males, carry the majority of serum cholesterol in HDL and absolute amounts of HDL increase with cholesterol feeding. Males have more atherosclerotic disease than females (90).

Histologically, quail atherosclerotic lesions are similar to human lesions. Lesions are characterized by massive lipid deposition, myofibroblastic proliferation, and in order quail fibrocelluar lesions and adherent thrombi (92,96). Calcification, severe coronary atherosclerosis and death from myocardial infarction have also been reported (92).

Atherogenic responses vary due to genetics of quail at different laboratories (92). Japanese quail have been selectively bred into strains susceptible and resistant to atherosclerosis (91). Macroscopic lesions in the susceptible strain are similar to early fatty streaks in man (97). These strains should be useful in providing etiological information on atherosclerosis (97). These selectively bred strains were not utilized in this study.

CHAPTER III

MATERIALS AND METHODS

Design

Male Japanese quail (Coturnix coturnix japonica) were fed from hatching to eight weeks of age with Ralston Purina Game Bird Startena. At six weeks demineralized water was given in watering jars in place of tap water. At eight weeks of age twenty birds were sampled to serve as pre-diet controls.

The remaining eighty birds were weighed, banded and randomly assigned to one of four dietary treatments which varied by protein source and calcium level. The quail were allowed food and demineralized water ad libitum throughout the study. After eight weeks on experimental diets the quail were terminated.

Quail were housed in metal batteries in a room with controlled temperature (18°C) and constant light. The top four tiers of a 58" x 37.5" x 72" chick battery were utilized. Each tier of the battery was divided into four pens with dimensions of 36" x 18.75 "x 9.5" (5.01 square feet). Five birds from each diet treatment were randomly assigned to one pen per tier, for a total of four pens per diet with one diet represented on each level of the battery.

Diets

Diet Composition is given in Table 1. Diets varied by protein source (soy protein or casein) and calcium content. Diets supplied 24% crude protein regardless of source. The low calcium diets met the nutrient requirement (0.8% calcium) so no calcium deficiency existed (98). High calcium diets supplied twice the requirement (1.6%) for nonbreeding male quail. Calcium was added as calcium carbonate and dicalcium phosphate.

Corn oil, an unsaturated fat, was used. All diets had cholesterol added at 0.5% to provide a ration which is known to be cholesterolemic, at 24% crude protein, to Japanese quail (99).

Sample Collection

Approximately five milliliters of blood was collected from each animal by decapitation. Twenty quail were sacrificed before experimental diets began. After eight weeks on treatment diets the remaining birds were bled.

Blood was collected in 50 milliliter beakers containing 50 microliters of ten percent ethylene diamine tetraacetic acid (EDTA) with a pH of 7.4. Samples were mixed by swirling, transferred to glass tubes and kept on ice until centrifugation.

Tubes were centrifuged at 1000 G for five minutes. Plasma was removed and put in capped plastic vials and stored at -28°C until analyzed.

TABLE I.

EXPERIMENTAL DIETS (PERCENT)

	SOY + 0.8% CALCIUM	SOY + 1.6% CALCIUM	CASEIN + 0.8% CALCIUM	CASEIN + 1.6% CALCIUM
CORNSTARCH	57.43	53.51	59.82	55.86
SOY PROTEIN*	28.43	28.48	0.0	0.0
CASEIN*	0.0	0.0	25.26	25.26
GLISTA SALTS*	5.37	5.37	5.37	5.37
POLYOLEFIN	3.0	3.0	3.0	3.0
DI CALCIUM PHOSPHATE	2.15	5.62	3.35	6.86
CORN OIL	2.0	2.0	2.0	2.0
CALCIUM CARBONATE	0.87	1.32	0.50	0.95
CHOLESTEROL	0.50	0.50	0.50	0.50
VITAMIN PREMIX	0.20	0.20	0.20	0.20

*Product analysis in Appendixes A, B, and C

Sample Analysis

Analysis of Total Cholesterol

Plasma samples were thawed in a 40°C water bath. Thawed samples were vortexed for ten seconds. The following procedure, based on the Lieberman-Burchard reaction (100), was used:

(1) Twenty µl of sample was placed in a twenty-milliliter test tube

(2) 0.2 ml of acetic acid-chloroform (3:2) reagent was added

(3) 5.0 ml of chloroform-acetic anhydride reagent (10:3) was added and mixed briefly

(4) 0.2 ml of reagent grade concentrated sulfuric acid was added then immediately and thoroughly vortexed

(5) Samples were centrifuged at 1000 G for ten minutes. The clear supernatant was decanted into 12 x 75 mm fluorometer tubes

(6) Forty minutes after the addition of sulfuric acid fluorescence was read using a Turner Fluorometer Model 111.

Plasma samples, blanks and aqueous standards (Sigma Chemical Company catalog #350-11) were run simultaneously.

Analysis of HDL Cholesterol

Serum LDL and VLDL were precipitated from plasma by Mg^{++} phosphotungstate and removed by centrifugation (101). The cholesterol associated with the soluble HDL fraction remains in the supernatant and is

quantified using the fluorometric method described for analysis of total cholesterol.

The procedure for separating HDL is:

(1) 0.4 ml of plasma was placed in a 15 ml conical centrifuge tube

(2) 0.05 ml of HDL precipitating reagent (Mg^{++} phosphotungstate) was added and mixed by vortexing

(3) Tubes were then centrifuged at 2000 G for five minutes to obtain a clear supernatant

(4) The supernatant was then analyzed for total cholesterol using the procedure described in "Analysis of Total Cholesterol" above.

Statistical Analysis

Data was analyzed using analysis of variance. Where appropriate pairs of means were compared using the student's test (102).

CHAPTER IV

RESULTS

Plasma Total Cholesterol

Figure 1 shows plasma total cholesterol and HDL in mg/dl for the commercial ration control and the four treatment diets. The control commercial diet represents the average plasma cholesterol concentrations of eight week old birds (169.4 ± 8.19 mg/dl) before being placed on experimental diets. Post-treatment total plasma cholesterol levels were significantly higher (P = .01) than pre-treatment diets.

Plasma total cholesterol in mg/dl, from highest to lowest levels, averages 296.20 ± 22.34, 234.34 ± 19.05, 231.79 ± 14.81, and 208.50 ± 15.15 for casein-low calcium, casein-high calcium, soy-low calcium and soy-high calcium diets respectively. These means are statistically different (P = .05) with the exception of the casein-high calcium and the soy-low calcium values, which are very similar (234.34 ± 19.05 and 231.79 ± 14.81 mg/dl).

Total plasma cholesterol was significantly different (P = .05) for birds consuming vegetable and animal proteins. The soy protein diet, at both calcium levels, average 219.79 ± 10.64 mg/dl compared to the more hypercholesterolemic casein diet which averaged 264.48 ± 15.27 mg/dl over both calcium levels.

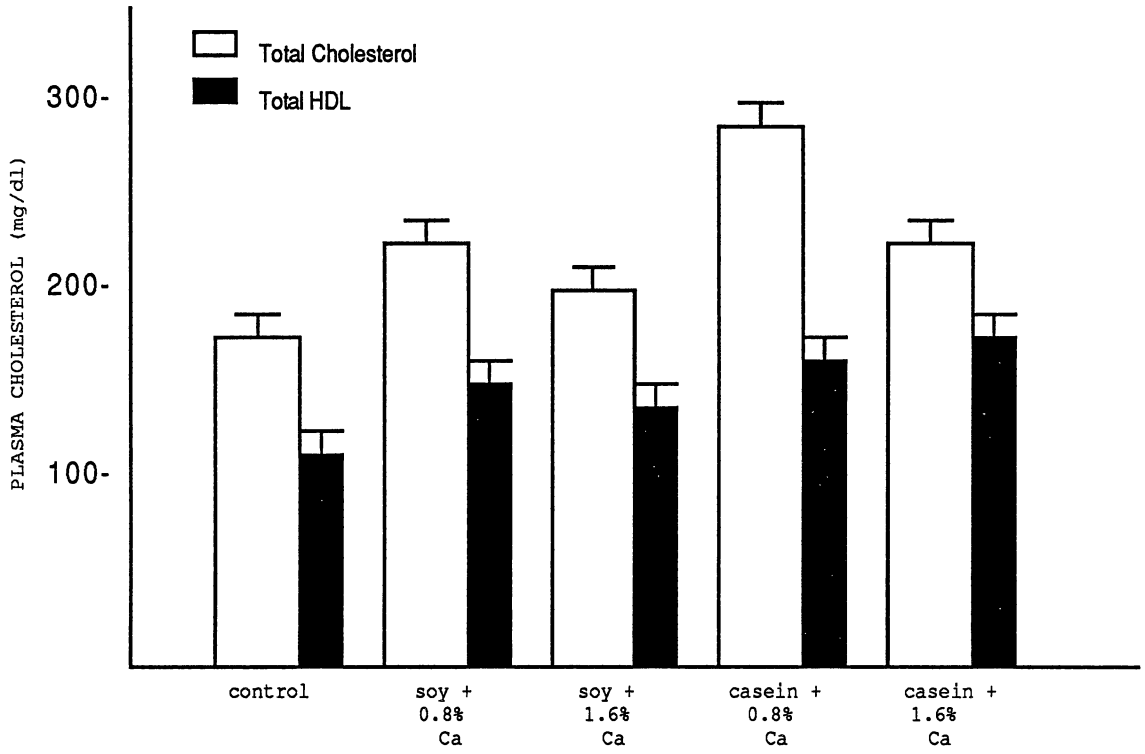

Figure 1. Plasma cholesterol and HDL concentrations in mg/dl. Control birds (n = 20) were sacrificed at 8 weeks of age after consuming commercial ration from hatching. Soy-low calcium (n = 17), soy-high calcium (n = 18), casein-low calcium (n = 19) and casein-high calcium (n = 20) were sacrificed at 15 weeks of age after 17 weeks on treatment diets. Mean cholesterol concentrations were significantly different at the one percent level. No significant differences in HDL concentration exist.

The low calcium diets, regardless of protein source, averaged significantly higher ($P = 0.05$) total plasma cholesterol levels (265.79 ± 14.57 mg/dl) than the high calcium diets (222.10 ± 12.35 mg/dl).

No consistent significant differences in plasma cholesterol resulted due to blocking (tier) effects ($F = 0.8$) so differences in cholesterol levels are attributed to diet effects.

Plasma HDL Cholesterol

Plasma HDL concentrations (Figure 1) paralleled changes in total plasma cholesterol but the means of plasma HDL concentrations were not significantly different. The pre-treatment control group had a mean HDL of 110.57 ± 8.97 mg/dl. Post-treatment HDL levels were higher, but not significantly so.

HDL concentrations in mg/dl for quail on treatment diets were 147.59 ± 9.78, 136.00 ± 12.06, 134.00 ± 9.28, and 130.47 ± 8.00 for birds on casein-low calcium, soy-low calcium, casein-high calcium and casein-low calcium respectively. Though these HDL changes correspond to fluctuations in plasma total cholesterol the magnitude of the changes is insufficient to be significantly different.

Vegetable protein diets, averaged over both calcium levels, resulted in plasma HDL levels of 133.16 ± 7.07 mg/dl. Birds consuming the animal protein diets, averaged over calcium levels, had HDL values of 138.05 ± 6.15. The higher HDL level on the casein diet is not significantly different than soy HDL.

The HDL cholesterol of the two low calcium diets averaged 136 ± 12.06 while the high calcium HDL was lower, 124.36 ± 10.37. These are not significantly different.

Mortality

Six quail died before termination of the experiment. One quail each died on weeks 2, 5 and 6 of the soy-low calcium diets. One quail each on the soy-high calcium diets died on weeks 3 and 5. One quail on the casein-low calcium diet died week six. Information on plasma lipid levels were not determined in these animals. No mortality occurred in the casein-high calcium diet.

Weight Changes

The mean weight of the control group was 136.0 ± 1.57 grams. Pre-treatment weights were 147.6 ± 2.7, 141.3 ± 2.3, 143.6 ± 2.6 and 142.4 ± 2.8 grams for diet groups as listed above. Quail on the low calcium diets gained an average of 5.0 grams for soy diets and 8.73 grams on casein diets. Birds on the high calcium diets showed a slight tendency to lose weight. Average losses were 4.58 grams on soy-high calcium and 2.15 grams on casein-high calcium diet.

CHAPTER V

DISCUSSION

Cholesterolemic Effects of Dietary Calcium and Protein

This study demonstrates a hypocholesterolemic effect of dietary calcium. Results are consistent with studies reporting hypercholesterolemic effects of animal proteins, like casein (70,71,75,83). Plasma HDL levels were not significantly altered by dietary treatment.

The casein-low calcium diet was most hypercholesterolemic; soy-high calcium was the least with approximately seventy percent lower plasma total cholesterol.

The data indicated that doubling the nutrient requirement level of calcium in a casein diet will lower plasma cholesterol to levels comparable to those resulting from a soy protein diet with no supplemental calcium. Additional dietary calcium lowered plasma cholesterol levels regardless of protein source.

The lower plasma total cholesterol in the commercial diet may be due to its higher protein content. It contained 30% crude protein. The experimental diets had 24% crude protein. A hypocholesterolemic effect of high dietary protein in high cholesterol rations has been reported in Japanese quail (78).

Possible Mechanisms

This study did not address mechanisms of the hypocholesteroemic effect of dietary calcium. Several possible mechanisms suggested by other investigators are discussed below.

Little information is available on the role of calcium in lipid metabolism (49). The mode of calcium induced hypocholesterolemia could be due to changes in cholesterol synthesis, absorption, conversion to bile acids, or changes in lipoprotein metabolism (37). Intravenous infusion of ionized calcium does not change serum cholesterol levels in humans (51). Many researchers think that calcium exerts its cholesterol lowering effects in the intestine, either by increased excretion of bile acids (46,51,52) or formation of insoluble complexes (42,49,52).

Dietary calcium may act by sequestering bile acids and suppressing enterohepatic circulation of cholesterol; this is the mechanism of the drug cholestyramine (51). Fleischman et al (44) found a three fold elevation in bile acid excretion in calcium supplemented rats corresponding to a decrease in serum and tissue cholesterol, including a 17% decrease in liver cholesterol. The liver is where cholesterol is oxidized to bile acids. Thus, increased fecal bile salt excretion could lower the cholesterol pool of the body. The degree of calcium binding of bile acids is uncertain (50).

Bhattacharyya (53) found no support for the hypocholesterolemic action of calcium mediated by increased excretion of bile acids in man. No change in fecal bile acid excretion occurred in polyunsaturated fat diets at two calcium

levels. Wells and Cooper (42) reported the failure of calcium to inhibit cholesterol absorption when added to fat free diets containing bile salts in rats.

A relationship exists between the amount of dietary calcium and fat absorption (50,55,59,60). Addition of calcium decreases digestibility of dietary fats, especially saturated fats (59).

When fat was omitted from rat's diets calcium did not inhibit cholesterol absorption suggesting calcium acts by forming insoluble complexes with phospolipids or fatty acids, inhibiting cholesterol absorption (42).

Fecal calcium excretion increased with increasing dietary calcium equally with saturated and unsaturated fat diets in rats (43). Bhattacharyya (53) observed increased elimination of neutral steroids in men at varying calcium levels, only with polyunsaturated fats. No correlation between serum cholesterol changes corresponded with changes in neutral steroid excretion in individuals.

In rats, a large increase in fecal lipids was not necessary for the cholesterol lowering effect of dietary calcium. Higher calcium levels did not decrease the degree of unsaturation of serum lipids so the mode of action may not be attributable to a change in the degree of unsaturation of absorbed fatty acids (44).

Human calcium supplementation increased both fecal fat and cholesterol excretion with no drop in serum total cholesterol. Calcium did appear to effect cholesterol metabolism.

Supplemented as calcium glycerophosphate, total neutral steroid excretion was unchanged while coprostanol excretion decreased, suggesting decreased bacterial conversion of cholesterol to coprostanol. In rats, bile acids

inhibit conversion of cholesterol to coprostanol and coprostanone by colon bacteria. Calcium reacting with bile acids result in insoluble bile salts, which in high amounts, depress the above mentioned bacterial conversion, perhaps accounting for the increased cholesterol excretion seen (52). Calcium given as skim milk resulted in increased neutral steroid and bile acid excretion (52).

In goats, dietary calcium was not shown to influence lipid absorption. No change in percent fecal lipid or fecal weight was observed in calcium carbonate supplemented animals through a slight, statistically insignificant, increase in total fecal lipids was observed (46).

Calcium interference with cholesterol absorption was not seen in a human study where fecal neutral steroid did not change with changing calcium content in the diet. High calcium diets slightly increased fecal saturated fatty acids but observed serum cholesterol reductions could not be explained by decreased fat absorption alone. If it is assumed that the fats must be absorbed to alter serum cholesterol, some other mechanism(s) must be acting (53).

A 25% lowering of serum total cholesterol with calcium supplementation resulted in reversal of the cholesterol:phospholipid ratio from 1.04 to 0.92. It was assumed HDL increased because as the ratio of phospholipid to other lipids increase, the density of serum lipoproteins increase (19).

Another human study saw a decreased total cholesterol:HDL ratio in high calcium control diets but a decreased ratio in skim milk diets. HDL could have been diverted to bile; bile cholesterol is derived from HDL (40).

In rabbits, a high calcium diet alters the free cholesterol:total cholesterol ratio suggesting calcium effects hepatic lipoprotein syntesis or alters enzymes

involved in free and esterified cholesterol regulation. Phospholipid total cholesterol ratios in the liver changed with dietary calcium levels indicating dietary calcium may participate in the regulation of tissue phospholipids and total cholesterol (20).

More information on the interaction of dietary calcium with mechanisms involved in cholesterol absorption, excretion and metabolism before calcium's hypocholesterolemic mechanism is understood.

Summary

(1) An increased level of dietary calcium was hypocholesterolemic in quail fed a diet with 0.5% added cholesterol.

(2) Casein was more hypercholesterolemic than soy protein, except when supplemented with dietary calcium.

(3) Plasma HDL did not change significantly due to dietary manipulation.

LITERATURE CITED

(1) National Institutes of Health Consensus Development Conference Statement. 1985. Lowering Blood Cholesterol. Nutr. Today. 20:13-16.

(2) The Lipid Research Clinics Program. 1984. The Lipid Research Clinics Coronary Primary Prevention Trial Results. I. Reduction in the Incidence of Coronary Heart Disease. JAMA 251(3):351-357.

(3) Kannel, W.B., J.T. Doyle, A.M. Ostfeld, C.D. Jenkins, L. Kuller, R.N. Podell, and J. Stamler. 1984. Optimal resources for primary prevention of atherosclerotic diseases. Circ. 70:155A-205A.

(4) Levy, R.I. 1981. Review: declining mortality in coronary heart disease. Arteriosclerosis 1:312-325.

(5) Naito, H.K. 1980. "Role of specific nutritional components on plasma lipids, lipoproteins and coronary heart disease." In: Nutritional Elements and Clinical Biochemistry. Plenum Press, New York, pp. 277-316.

(6) Is total serum cholesterol level a reliable indicator of latent risk of coronary heart disease? 1984. Nutr. Today. 20:11-12.

(7) The Lipid Research Clinics Program. 1984. The Lipid Research Clinics Coronary Primary Prevention Trial Results. II. The relationship of reduction in incidence of coronary heart disease to cholesterol lowering. JAMA 251(3)365-374.

(8) Walker, W.J. 1983. Changing U.S. lifestyle and declining vasular mortality - a retrospective. New Eng. J. Med. 308(11)649-651.

(9) Connor, W.E. 1979. The relationship of hyperlipoproteinnnemia to atherosclerosis: the decisive role of dietary cholesterol and fat. In: The Biochemistry of Atherosclerosis, Marcel Dekker, Inc. New York.

(10) Kritchevsky, D. 1982. Atherosclerosis: dietary factors other than the usual lipids. Fed. Proc. 41:2790-2791.

(11) Sirtori, C.R. 1982. "Experimental and clinical studies on the cholesterol lowering activity of the soybean protein diet." In: Soy Protein in the Prevention of Atherosclerosis. MTP Press, Hinghem, MA, pp. 13-20.

(12) Kummerow, F.A. 1981. Dietary recommendations to reduce cholesterol consumption may have undesirable consequences. Arterial Wall (7):3-5.

(13) Goodwin, J.S., A.G. Leonard, E.M. Hooper, P.J. Garry. 1985. Concern about cholesterol and its association with diet in a group of healthy elderly. Nutr. Res. 5:141-148.

(14) Perry, H.M. 1973. Minerals in cardiovascular disease. J. Amer. Diet. Assoc. 62:631-642.

(15) Crawford, M.D., M.J. Gardner, J.N. Morris. 1971. Changes in water hardness and local death rates. Lancet (7720):327-329.

(16) Hankin, J.H., S. Marsen, N.F. Goldsmith. 1970. Contribution of hard water to calcium and magnesium intakes in adults. J. Amer. Diet. Assoc. 56:212-224.

(17) Van Barneveld, A.A., C.J. van den Hamer. 1985. Drinking water hardness, trace elements and cardiovascular diseases: main effects of Ca and Mg on Metabolism of Mn, Pb, and Cd in mice. Nutr. Res. I:345-349.

(18) Higgins, I.T.T. 1975. Importance of epidemiological studies relating to the hazards of food and environment. Br. Med. Bull. 31(3):231-232.

(19) Bierenbaum, M.L., A.I. Fleischman, R.I. Raichelson. 1972. Long term human studies on the lipid effects of oral calcium. Lipids 7:202-206.

(20) Iacono, J.M. 1974. Effect of varying the dietary level of calcium on plasma and tissue lipids of rabbits. J. Nutr. 104:1165-1171.

(21) Albanese, A.A., A.H. Edilson, M.L. Woodhull, E.J. Lorenze, E.H. Wein and L.A. Crto. 1973. Effect of a calcium supplement on serum cholesterol calcium, phosphorus and bone density of "normal, healthy" elderly females. Nutr. Rept. Intr. 8:119-130.

(22) McCarron, D.A., C.D. Morris, and H.J. Henry, and J.L. Stanton. 1984. Blood pressure and nutrient intake in the United States. Science 224:1392-1395.

(23) Parrott-Garcia, M. and D.A. McCarron. 1984. Calcium and Hypertension. Nutr. Rev. 42:205-213.

(24) Harlan, W.R., A.L. Hull, R.L. Schmouder, J.R. Landis, F.G. Thompson and F.A. Larkin. 1984. Blood pressure and nutrition in adults. Amer. J. of Epidemiology 120:17-28.

(25) Wasserman, R.H. Ca and P interactions in nutrition and physiology. Fed. Proc. 19:636-642.

(26) Martin, D.W. 1983. "Water and Minerals". In: Harper's Biochemistry. Lange Medical Publ., Los Altos.

(27) Moon, J.Y. 1972. Factors affecting arterial calcification associated with atherosclerosis. Atherosclerosis 16:119-126.

(28) Allen, L.H. 1982. Calcium bioavailability and absorption: a review. Am. J. Clin. Nutr. 35:783-808.

(29) Levine, B.S., M.W. Walling and J. Coburn. 1982. "Intestinal absorption of calcium. Its assessment, normal physiology, and alterations in various disease states". In: Disorders of Mineral Metabolism, Volume II. Academic Press, Orlando.

(30) Perry, H.M. 1973. Minerals in cardiovascular disease. J. Am. Diet Assoc. 62:631-642.

(31) Schroder, H.A. 1960. Relation between mortality from cardiovascular disease and treated water supplies: variations in status in 163 largest municipalities of the United Staes. JAMA 172:1902.

(32) Belizan, J.M., J. Villar, D. Pineda, A.E. Gonzolez, E. Sainz, G. Garrera and R. Sibrian. 1983. Reduction of blood pressure with calcium supplementation in young adults. JAMA 249(9)1161-1166.

(33) Thakur, C.P. and A.N. Jha. 1981. Influence of milk, yoghurt and calcium in cholesterol induced atherosclerosis in rabbits. Atherosclerosis 39:211-215.

(34) Hussi, E., T.A. Miettinen, A. Ollus, E. Kostiarnen, C. Ehnholm, B. Haglund, J.K. Huttunen and V. Menninen. 1981. Lack of serum cholesterol-lowering effects of skim milk and buttermilk under controlled conditions. Atherosclerosis 39:267-272.

(35) Mann, G.V. and A. Spoerry. 1974. Studies of a surfactant and cholesterolemia in the Maasai. Am. J. Clin. Nutr. 27:464-469.

(36) Hepner, G., R. Fried, S. St. Jeor, L. Tusetti and R. Moren. 1979. Hypocholesterolemic effect of yogurt and milk. Am. J. Clin. Nutr. 32:19-24.

(37) Kritchevsky, D., S.A. Tepper, M. Mueller, S. Czarnecki, and D.M. Klurfeld. 1983. Pre- and Post- weaning consumption effects on lipid metabolism in rats. Atherosclerosis 47:241-250.

(38) Rossouw, J.E., E. Burger, P. van der Vyver and J. Ferreira. 1972. The effect of skim milk, yoghurt and full cream milk on human serum lipids. Am. J. Clin. Nutr. 34:351-356.

(39) Kein, N.L., J. Marlett and C. Amundson. 1982. The cholesterolemic effect of skim milk in young men consuming controlled diets. Nutr. Res. 1:429-442.

(40) Howard, A.N. and J. Marks. 1979. Effect of milk products on serum cholesterol. Lancet :957.

(41) Wells, W.W. and S. Cooper. 1958. Coprostanol formation II. The opposing action of dietary lactose and calcium on cholesterol absorption. Arch. Biochem. Biophys. 75:273-279.

(42) Yacowtiz, H., A. Fleischman, R. Amsden, and M. Bierenbaum. 1967. Effects of dietary calcium upon lipid metabolism in rats fed saturated or unsaturated fat. J. Nutr. 92:389-392.

(43) Fleischman, A., H. Yacowitz, T. Hayton, and M. Bierenbaum. 1966. Effects of dietary calcium upon lipid metabolism in mature male rats fed beef tallow. J. Nutr. 88:255-260.

(44) Fleischman, A.I., M. Bierenbaum, and P. Lenz. 1972. The hypolipidemic effect of calcium containing compounds and vitamin D_2 on the rat. Lipids 7:263-266.

(45) Hines, T.G., N. Jacobson, D. Beitz and E.T. Littledike. 1985. Dietary calcium and vitamin D: risk factors in the development of atherosclerosis in young goats. J. Nutr. 115:167-178.

(46) Iacono, J.M. and C. Ammerman. 1966. The effect of calcium in maintaining normal levels of serum cholesterol and phospholipids in rabbits during acute starvation. J. Nutr. 18:197-202.

(47) Yacowitz, H., A. Fleischman, and M.L. Bierenbaum. 1965. Effects of oral calcium upon serum lipids in man. British Med. J. 1:1352.

(48) Carlson, L., A. Olsson, L. Oro, S. Rossner. 1971. Effects of oral calcium upon serum cholesterol and triglycerides in patients with hyperlipidemia. Atherosclerosis 14:391-400.

(49) Mitchell, W.D., T. Fyfe, and D. Smith. 1968. The effect of oral calcium on cholesterol metabolism. J. Athero. Res. 8:913-922.

(50) Bhattacharrya, A.K., C. Thera, J. Anderson, F. Grande and A. Keys. 1969. Dietary calcium and fat: effect on serum lipids and fecal excretion of cholesterol and its degradation products in man. Am. J. Clin. Nutr. 22(9)1161-1174.

(51) Yacowitz, H., A. Fleischman, M. Bierenbaum, and D. Kritchevsky. 1971. Effects of increased dietary calcium on atherosclerosis in rabbits. Trans. NY Acad. Sci. 33:344-350.

(52) Drenick, E.J. 1961. The influence of calcium and other soap-forming substances on fecal fat. Gasteroenterology 41:242-244.

(53) Carey, M.C. and D.M. Small. 1970. The characteristics of mixed micellar solutions with particular reference to bile. Am. J. Med. 49:590-608.

(54) Siperstein, M., I. Chaikoff and W. Reinhardt. 1952. V. Obligatory function of bile in intestinal absorption of cholesterol. J. Biol. Chem. 198:111-114.

(55) Nomani, M., C. Giaquinto, J. Lim, D. Buckner, R. Jamil, N. Rodriguez, and A. Watne. 1985. Relationship of serum cholesterol level with the dietary fat, fecal bulk and steroid output. Nutr. Rept. Inter. 31:321-328.

(56) Cheng, A., M. Morehouse and H. Deuel. 1949. The effect of the level of dietary calcium and magnesium on the digestibility of fatty acids, simple triglycerides, and some natural and hydrogenated fats. J. Nutr. 37:237-250.

(57) Fedde, M., P. Waibel and R. Burger. 1959. Factors effecting the digestability of certain dietary fats in the chick. Poult. Sci. 38(1203) abstract.

(58) Whitehead, C., W. Dewar and J. Downie. 1971. Effect of dietary fat on mineral retention in the chick. Br. Poult. Sci. 12:249-254.

(59) Davison, K.L. and W. Woods. 1962. Effect of calcium and magnesium upon digestibility of a ration containing corn oil by lambs. J. Anim. Sci. 22:27-29.

(60) Beare-Rogers, J. 1984. Dietary fatty acids and cholesterol. Nutr. Res. 4:533-535.

(61) Thatcher, C., N. Jacobson, J. Young and M.J. Richard. 1984. Effects of sources of dietary fat and protein on tissue cholesterol. Nutr. Res. 4:1013-1024.

(62) Byers, S. and M. Friedman. 1958. Bile Acids metabolism, dietary fats, and plasma cholesterol levels. P.S.E.B.M. 98:523-524.

(63) Myasnikov, A. 1958. Influence of some factors on development of experimental cholesterol atherosclerosis. Circ. 17:99-113.

(64) McCarron, D.A. 1982. Low serum concentrations of ionized calcium in patients with hypertension. New Eng. J. Med. 307(4):227-228.

(65) Sunderrajan, S. and J. Bauer. 1984. Oral calcium supplementation does not alter blood pressure or vascular response in normotensive men. Circ. 70(II) II-130.

(66) Ignatowski, A. 1909. Uber die wirkung des tierischen eiveisses auf die aorta und die parenchymatose organe der kaninchen. Virchows Arch. Pathol. Anat. Physiol. Klin. Med. 198:248.

(67) Meeker, D. and H. Kesten. 1941. Effect of high protein diets on experimental atherosclerosis in rabbits. Arch. Pathol. 31:147.

(68) Carroll, K. 1982. Hypercholesterolemia and atherosclerosis effects of dietary protein. Fed. Proc. 41:2792-2796.

(69) Huff, M. and K. Carroll. 1980. Effects of dietary protein on turnover, oxidation and absorption of cholesterol, and on steroid excretion in rabbits. J. Lipid Res. 21:546-558.

(70) Roy, D. and B. Schneeman. 1981. Effect of soy protein, casein, and trypsin inhibitor on cholesterol, bile acids and pancreatic enzymes in mice. J. Nutr. 111:878-885.

(71) Cohn, J., W. Kimpton and P. Nestel. 1984. The effect of dietary casein and soy protein on cholesterol and very low density lipoprotein metabolism in the rat. Atherosclerosis 52:219-231.

(72) West, C.E., A. Beynen, A. Terpstra, K. Scholz, K. Carroll, and C. Woodward. 1983. The nature of dietary protein and serum cholesterol. Atherolsclerosis 46:252-254.

(73) Hevia, P. and W. Visek. 1979. Dietary protein and plasma cholesterol in chickens. J. Nutr. 109:32-38.

(74) Terpstra, A., J. Schutte, and C. West. 1983. Prevention of hypercholesterolemia in cholesterol-fed chickens by high-casein and high-soybean protein diets. Atherosclerosis 46:95-104.

(75) Hursey, Z.W., C.G. Beames, Jr., and C.V. Maxwell. 1985. Effect of protein quality and quantity upon plasma lipids of quail. Fed. Proc. abstract 4359.

(76) van Raaij, J., M. Katan, C. West, and J. Hautvast. 1982. Influence of diets containing casein, soy isolate, and soy concentrate on serum cholesterol and lipoproteins in middle-aged volunteers. Am. J. Clin. Nutr. 35:925-934.

(77) Grundy, S. and J. Abrams. 1983. Comparison of actions of soy protein and casein on metabolism of plasma lipoproteins and cholesterol in humans. Am. J. Clin. Nutr. 38:245-252.

(78) van Raay, J. 1983. Influcne of human diets containing casein, soya protein isolate and soya protein concentrate on serum cholesterol and lipoproteins in humans, rabbits and rats. Neth. Milk Dairy J. 37:101-104.

(79) Sacks, F., J. Breslow, P. Woods and E. Kass. 1983. Lack of an effect of dairy protein (casein) and soy protein on plasma cholesterol of strict vegetarians. An experiment and a critical review. J. Lipid Res. 24:1012-1020.

(80) Shorey, R., B. Bazan, G. Lo, and F. Steinke. 1981. Determinants of hypocholesterolemic response to soy and animal protein-based diets. Am. J. Clin. Nutr. 34:1769-1778.

(81) Barth, C., M. Pfeuffer, and G. Hahan. 1984. Influence of dietary casein or soy protein on serum lipids and lipoproteins of monkeys (Macaca fascicularis). Ann. Nutr. Metab. 28:137-143.

(82) Kritchevsky, D. 1983. Dietary Protein and Atherosclerosis. Atherosclerosis 49:209-210.

(83) van der Meer, R. 1983. Is the hypocholesterolemic effect of dietary casein related to its phosphorylation state? Atherosclerosis 49:339-340.

(84) Grundy, S. 1983. Absorption and metabolism of dietary cholesterol. Ann. Rev. Nutr. 3:71-96.

(85) Tradwell, C. and G. Vanhouny. 1968. Cholesterol Absorbtion. In: Handbook of Physiology III (6):1407-1438.

(86) Martin, D., P. Mayes and V. Rodwell. 1983. Harper's Review of Biochemistry,m 19th edition. Lange Medical Publications, Los Altos.

(87) Lewis, B. 1980. "The LDL theory and the HDL hypothesis." In: Diet and Drugs in Atherosclerosis, Raven Press, New York.

(88) Witzum, J. and G. Schonfeld. 1979. High density lipoproteins. Diabetes 28:326-333.

(89) Shih, J.C.H. 1983. Avian models for reserach in atherosclerosis and cholesterol metabolism. Fed. Proc. 42:2475.

(90) Wu, T.C. and W. Donaldson. 1982. Effect in cholesterol feeding on serum lipoproteins and atherosclerosis in atherosclerosis-suseptible and atherosclerosis-resistant Japanese quail. Poult. Sci. 61:2407-2414.

(91) Chapman, K., W. Stafford and C. Day. 1975. "Produced by selective breeding of Japanese quail animal model for experimental atherosclerosis." In: Advances in Experimental Medicine and Biology. Volume 67. Plenum Press, NY, pp. 347-356.

(92) Shih, J.C.H. 1983. Atherosclerosis in Japanese quail and the effect of lipoic acid. Fed. Proc. 42:2494-2497.

(93) Donaldson, W.E. 1982. Atherosclerosis in cholesterol fed Japaneses quail: evidence for amelioration by dietary vitamin E. Poult. Sci. 61: 2097-2102.

(94) Sutton, C.D., W. Muir and J. Begin. 1981. Effect of fiber on cholsterol metabolism in the Coturnix quail. Poult. Sci. 60:812-817.

(95) Subcommittee on Avian Standards, Institute of Lab Animal Resources. 1969. Coturnix: Standards and Guidelines for the Breeding, Care and Management of Laboratory Animals. National Acad. of Science Pub. 1703. washington, D.C.

(96) Shih, J.C.H., E. Pullman, and K. Kao. 1983. Genetic selection, general characterization of histology of atherosclerosis-suseptible and -resistant Japanese quail. Atherosclerosis 49:41-53.

(97) Soret, M.G., T. Peterson, K. Chapman, W. Stafford, C. Day and E. Block. Morphological observations in experimental atherosclerosis in SEA Japanese quail. In: Advances in Expt. Med. and Biol. Vol. 67, Plenum Press, New York, pp. 357-376.

(98) National Research Council Subcommittee on Poultry Nutrition. 1984. Nutrient requirements of poultry. The National Academy Press, Washington, D.C.

(99) Zola Hersey, unpublished data.

(100) Sigma Technical Bulletin #351.

(101) Sigma Technical Bulletin #350-HDL.

(102) Snedecor, G.W. and W.G. Cochran. 1980. Statistical Methods, Seventh Edition. The Iowa State Univ. Press, Ames.

APPENDIXES

APPENDIX A

SOYBEAN PROTEIN ANALYSIS

Protein, N x 6.25 (as is basis: 90%+
Moisture: 4.8%
Water - soluble protein
(as % of total protein): 75%
Crude fiber: 0.25%
Ash: 4.0%
pH (1:10 aqueous dispersion): 6.9
Phosphorus: 0.9%
Sodium: 1.5%
Calcium: 0.3%
Iron: 0.02%
Potassium: 0.1%
Magnesium: 0.1%

Amino Acid Composition of Soybean Protein, Grade II
(g. Amino Acid/16 g. N)
Lysine: 5.7
Methionine: 0.9
Cystine: 1.2
Threonine: 3.6
Leucine: 7.8
Isoleucine: 4.6
Phenylalanine: 5.2
Tyrosine: 3.6
Valine: 4.5
Tryptophane: 1.1

Soybean protein supplied by:
United States Biochemical Corporation
P.O. Box 22400, Cleveland, OH 44122

APPENDIX B

Elemental	Analysis of "Kerrynor Brand" Irish Casein
P	0.123%
K	<0.0500%
Ca	0.0330%
Mg	<0.0050%
Na	0.0268%
A1	<5.00 ppm
Ba	<1.00 ppm
Fe	<2.50 ppm
Sr	<0.500 ppm
B	0.912 ppm
Cu	0.722 ppm
Zn	9.09 ppm
Mn	<1.00 ppm
Cr	<1.50 ppm
Cd	0.05 ppm

Product analysis provided by:
Eire Casein Company, Inc.
P.O. Box 648
Eire, Illinois 61250

APPENDIX C

CALCIUM AND PHOSPHORUS DEFICIENT CHICK MINERAL MIX

TD 84456

		g/kg
Sodium Chloride	NaCl	164.0894
Potassium Bicarbonate	$KHCO_3$	192.9423
Magnesium Sulfate	$MgSO_4 \cdot 7H_2O$	65.2626
Manganese Sulfate	$MnSO_4 \cdot H_2O$	12.121
Ferric Citrate		9.3222
Zinc Carbonate		1.8659
Cupric Sulfate	$CuSO_4 \cdot 5H_2O$	0.3724
Boric Acid		0.1676
Sodium Molybdate	$Na_2MoO_4 \cdot 2H_2O$	0.1676
Potassium Iodide	KI	0.7467
Cobalt Sulfate	$CoSO_4 \cdot 7H_2O$	0.0186
Sodium Selenite	Na_2SeO_3	0.0037
Corn Starch		552.92

Designed for use at 5.37% of chick diet. This formula is a modification of TD 73007. The lot number used was 403293.

This Glista Salt mineral mix was formulated by:
TEKLAD
P.O. Box 4200
Madison, WI 53771

VITA

Lori L. Johnson Hake

Candidate for the Degree of

Master of Science

Thesis: THE EFFECT OF DIETARY CALCIUM, CASEIN, AND SOY ON PLASMA CHOLESTEROL IN THE JAPANESE QUAIL (COTURNIX COTURNIX JAPONICA)

Major Field: Zoology

Biographical:

Personal Data: Born in Springfield, Illinois, April 23, 1958, the daughter of Raymond K. and Joanne D. Johnson. Married to Daniel R. Hake on May 25, 1985.

Education: Graduated from Shawnee Mission South High School, Overland Park, Kansas, in May, 1976; received Bachelor of Science Degree in May, 1981; completed requirement for Master of Science degree at Oklahoma State University in May, 1986; currently pursuing Doctor of Osteopathic Medicine degree at the Oklahoma College of Osteopathic Medicine and Surgery in Tulsa, Oklahoma.

Professional Experience: Teaching Assistant, Department of Zoology, Oklahoma State University, August 1984 to May 1985.

Professional Organizations: Oklahoma Osteopathic Association, American College of General Practitioners, Delta Omega, Sigma Sigma Phi.