COMPARATIVE EFFECT OF DIETARY ANIMAL AND VEGETABLE PROTEIN ON CHOLESTEROL GALLSTONE FORMATION AND BILIARY CONSTITUENTS IN THE HAMSTER

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We hereby recommend that the thesis prepared under our supervision by Susan Mahfouz entitled Comparative Effects of Dietary Animal and Vegetable Protein on Cholesterol Gallstone Formation and Biliary Constituents in the Hamster

be accepted as fulfilling this part of the requirements for the Degree of Master of Science.

Committee

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CHAPTER I

INTRODUCTION

Approximately 16 million Americans suffer from gallstone disease; 800,000 Americans develop gallstones each year. Gallstones hospitalize 500,000 persons annually with at least 60 per cent of these admissions requiring surgery. Cholecystectomy is the most common procedure in general surgery. The medical cost of gallstone disease in the United States alone approximates one billion dollars per year (1-3).

Human gallstones occur in two major types, cholesterol and pigment, and are classified according to their predominant components (4). Cholesterol stones are either white or yellow crystals which contain approximately 80 per cent cholesterol. In North America and Europe approximately 85 per cent of the gallstones are cholesterol stones. Pigment stones are brown or green in color, amorphous and may contain pure bilirubin and/or calcium bilirubinate. The formation of pure pigment stones is commonly associated with cirrhosis of the liver and hemolytic states. Pigment stones comprise 10 per cent of the gallstones in the western world although they are the major class of stone in the Far East and Africa. Mixed gallstones comprise five per cent

of the gallstones in the western world and are composed of cholesterol and bile pigments (2).

Risk factors have been defined with respect to the formation of cholesterol cholelithiasis (4). In man gallstone incidence increases with age; cholelithiasis is rare in persons under 20 years of age. After the fourth decade of life, however, approximately one out of four persons is afflicted with cholelithiasis and, after the seventh decade, the incidence may be as high as one out of two in some races. Gallstones are twice as common in females. Incidence increases with obesity, diabetes, pancreatitis and disorders of the liver (2).

On the basis of clinical and epidemiological data diet appears to play a significant role in the development of gallstone disease although the mechanism of action is not clearly defined. The relationship between dietary protein and gallstone formation is one of particular interest since proteins derived from animal sources seem to have a greater tendency to elevate plasma cholesterol than proteins derived from plant sources (5-7). This elevation of plasma cholesterol found with dietary animal proteins could play a role in gallstone formation since cholesterol gallstones are found when bile becomes supersaturated with cholesterol (8).

The purpose of this study was to examine the effect of

dietary proteins, namely casein, soybean or cottonseed, on cholesterol gallstone formation and the composition of biliary fluid in the hamster. The specific objectives of this study were to:

1. examine the effect of dietary proteins on cholesterol gallstone formation;
2. examine the effect of dietary proteins on the absolute and relative concentrations of the three primary biliary constituents bile acids, phosopholipid and cholesterol;
3. determine the effect of dietary proteins on total serum cholesterol;
4. determine if there is a correlation between the level of cholesterol in the plasma and bile and;
5. investigate whether the source and/or amount of carbohydrate present in the diet is related to cholesterol gallstone formation.

CHAPTER 2

REVIEW OF LITERATURE

Bile Metabolism and Functions

Bile, a mixture of both secretory and excretory products, is composed of water, bile salts, bile pigments, inorganic salts and lipid materials including lecithin and cholesterol. A small amount of protein, primarily in the form of plasma protein, is present in human bile. Human gallbladder bile is viscous, yellow-brown or greenish in color, bitter and has a neutral to slightly alkaline pH (2).

Bile acids, the major solid component of bile, are amphiphilic, detergent like molecules derived from the catabolism of cholesterol in the liver (4). In most mammalian species the primary bile acids are cholic and chenodeoxycholic acid. These bile acids are conjugated with the amino acids taurine and glycine with the ratio of glycine to taurine conjugates approximately 3:1, however, this ratio may be influenced by diet, hormones, disease and various micro-organisms. Conjugated bile acids combine with sodium or potassium ions producing bile salts which are subsequently secreted into the bile (2,9).

In the lower portion of the small intestine the pri-

mary bile acids are subjected to metabolic interaction with various microorganisms which result in the conjugated bile acid being hydrolyzed to a free bile acid. Additionally, the 7 alpha-hydroxy group is removed from cholic and chenodeoxycholic acid yielding deoxycholic and lithocholic acid respectively. These are classifed as secondary bile acids. Approximately 95 per cent of the secondary bile acids are absorbed by the gut, recirculated to the liver and resecreted as bile (10). This secretion, absorption, and resecretion is referred to as the enterohepatic circulation of the bile salts. Normally a bile salt will participate in 15 to 20 enterohepatic cycles before being excreted. The primary pathway for the excretion of bile salts is via the feces. Less than five per cent normally appears in the urine (2).

Bile pigments are degradative byproducts of hemoblobin, formed primarly in the liver and which contribute 15 to 20 per cent of the total biliary solids. Biliverdin is the first bile pigment which is formed and is rapidly reduced to bilirubin the chief pigment in human bile. Derivatives of bilirubin include urobilinogen, urobilin, bilifuscin and bilicyanin. As bilirubin enters the intestine it is reduced by bacteria to urobilinogen, some of which is excreted via the feces. The remainder is reabsorbed and recirculated to the liver where it is partially converted

to bilirubin and finally re-excreted into the bile as bilirubin and urobilinogen (2,9).

More than 90 per cent of the phospholipid in human bile is lecithin which comprises 0.02 to 0.05 per cent of the total biliary solids (11). Addition of lecithin to an aqueous solution of bile acids results in the formation of mixed micelles that have a greater ability to solubilize cholesterol than pure bile acid micelles. Human bile contains approximately one lecithin molecule for every three bile acid molecules (2,4).

The cholesterol content of human hepatic bile normally ranges from 0.04 to 0.16 per cent; however, the percentage in gallbladder bile is increased due to the absorption of water and salts by the mucosal cells of the gallbladder. The ability of bile to solubilize cholesterol is largely dependent upon the relative concentration of bile salts and lecithin to cholesterol. The normal ratio of cholesterol to bile salts ranges from 1:20 to 1:30; however, if this ratio falls below 1:13, cholesterol crystals precipitate which can ultimately result in the formation of cholesterol gallstone (2).

In man, bile is secreted continuously by the parenchymal cells of the liver at the rate of 500 ml/24 hours (12). Secretion of bile from the liver is stimulated by bile salts, secretin and the autonomic nervous system.

After formation, bile passes through the hepatic and cystic ducts into the gallbladder where it is concentrated and stored. The concentration of hepatic bile is necessary since the gallbladder's capacity for storage is limited to approximately 50 milliliters. Water and inorganic salts are absorbed through the lymphatic and blood vessels of the gallbladder; however, conjugated bile salts, bile pigments and lecithin are not absorbed to any appreciable degree (1). Absorption of significant quantities of free cholesterol may serve as a protective mechanism against cholesterol supersaturation of gallbladder bile (13). The resulting gallbladder bile may contain 10 times more solids than hepatic bile. This concentrating process enhances biliary cholesterol holding capacity and stability (14). In addition to the concentration and storage of the bile, the gallbladder reduces the alkalinity of the bile and equalizes pressure within the biliary duct system (2).

During fasting, the release of bile from the gallbladder into the duodenum is prevented by the tonic contraction of the sphincter of Oddi. The evacuation of gallbladder bile is stimulated by the presence of food in the intestine. When fatty substances, acids and/or partially digested proteins come in contact with the intestinal mucosa, cholecystokinin is activated which causes the gallbladder to contract with

the subsequent relaxation of the sphincter of Oddi (15). Contractions are occassionally induced by the taste or smell of food. Drugs have an effect on the evacuation of the gallbladder: epinephrine, vasopressin, histamine and methacholine stimulate evacuation; morphine and ergotamine inhibit evacuation (2,10).

When the walls of the gallbladder contract, bile is discharged via the cystic and common bile ducts into the upper portion of the small intestine. In the upper portion of the small intestine bile is partially responsible for alkalizing the intestinal contents. In the jejunum bile salts solubilize dietary triglyceride by forming a mixed micellar solution of bile salts, fatty acids and monoglycerides. The monoglycerides and fatty acids are readily absorbed from the micelle while the bile salts continue down the small intestine where they are absorbed primarily from the terminal ileum. The absence of bile results in the incomplete activation of pancreatic lipase and reduced absorption of fat and fat soluble vitamins (2).

The Etiology of Gallstone Disease

The process of gallstone formation is thought to occur in three stages: formation of saturated bile, nucleation or initiation of stone formation and the growth of the stone to a detectable size without first being discharged from

the gallbladder into the intestine (16). The following will be a discussion regarding the process of lithogenesis, the formation of saturated bile, which has received the greatest attention with regard to cholesterol cholelithiasis (4).

In a classic study, Admirand and Small (8) reported that the presence or absence of insoluble cholesterol in bile is determined by the relative concentration of bile salts, cholesterol, lecithin and other biliary constituents. This study made clear that the primary defect in cholesterol cholelithiasis is the formation of bile that is supersaturated with cholesterol. The study did not indicate whether the supersaturated bile was the result of cholesterol excess or a decrease in bile acids and/or phospholipid.

Normally the percentages of bile salts, lecithin and cholesterol in human bile are 70 to 80 per cent, 28 to 32 per cent and two to eight per cent respectively. When the concentration of bile salts falls below 40 per cent, or the cholesterol concentration rises above 10 per cent, cholesterol crystals precipitate possibly resulting in the formation of a gallstone. The incidence of gallstones with respect to the relative or absolute concentration of cholesterol, bile salts and lecithin has been illustrated in man (8,17) and animals (18,19).

Early studies concerning the mechanism of formation of supersaturated bile have reported that many patients

with the condition have a decreased bile acid pool (20). A decreased bile acid pool in patients with gallstones could result from an increased loss via the feces, eg. ileal disease; increased cycling of bile acids and decreased synthesis of bile acids. Patients with cholesterol gallstones have been reported to have a decreased level of the key enzyme, cholesterol-7 alpha-hydroxylase, which regulates the conversion of cholesterol to bile acids (21).

Alternatively, supersaturated bile could result from an increase in cholesterol synthesis or an increase in exogeneous cholesterol from dietary sources. The rate limiting step for cholesterol synthesis in the liver is the conversion of 3-hydroxy-3 methylglutaryl CoA to mevalonic acid which is governed by the enzyme HMG CoA reductase. Hepatic synthesis of this enzyme is increased in patients with gallstones (21,22). This suggests a pathogenic sequence of increased cholesterol synthesis resulting in the increased secretion and production of supersaturated bile.

Supersaturated bile could result from a decrease in phospholipid concentration; however, most investigators have observed that the relative concentration of phospholipid in the bile of cholesterol gallstone patients is normal (23,24). In contrast to the above findings, Kajiyama et al.

(25) recently reported that formation of lithogenic bile in hamsters was caused by a relative decrease in phospholipid concentration.

Bile supersaturated with cholesterol is frequently found in healthy individuals; without concomitant formation of gallstones (14,26). Bile of some individuals may contain inhibitors of nucleation and growth or lack of a pathogenic factor required for crystal nucleation. Nucleation may be influenced by bile acid structure, pH of the bile and the presence of agents capable of seeding such as bile pigments, mucoproteins, bacteria and calcium salts (4).

Dietary Effects on Gallstone Disease

Clinical and epidemiological data reveal that diet plays a significant role in the development of gallstone disease. Dietary factors including cholesterol (18,27,28), fat (29-31), carbohydrate (32-34) and excessive caloric intake (35-37) have all be implicated with respect to gallstone formation.

In 1952 Dam and Christensen (32) reported that cholesterol gallstones could be produced in hamsters by diet alone. The diet, referred to as the Dam Diet or Diet 284, consisted of 74.3 per cent sucrose, 20.0 per cent casein, 5.0 per cent mineral mix, 0.5 per cent vitamin mix and

0.2 per cent choline chloride. Gallstones were produced in 80 to 100 per cent of the hamsters within six to eight weeks. In a later study, Dam et al. (38) demonstrated that the replacement of sucrose with whole wheat, rolled oats or sorghum seeds markedly inhibited gallstone formation. Furthermore, gallstones that were originally induced with the Dam Diet could be dissolved by substituting a mixture of yeast and polised rice for a simple sugar sucrose (34). These observations gave support to the theory that dietary carbohydrate may influence the development of cholesterol cholelithiasis.

The addition of polyunsaturated fatty acids to the diet has been shown to counteract the formation of cholesterol gallstones. Three oils (lard, soybean, or cod liver) were added to the Dam Diet at a two per cent level. The Dam Diet produced gallstones in 84 per cent of the hamsters; however, the addition of lard, soybean oil or cod liver oil reduced cholesterol gallstones formation to sixty five and zero per cent of the animals respectively (39).

To date most of the research emphasizing effects of diet on gallstone formation has concentrated on dietary cholesterol and fat with some consideration being given to other dietary constituents such as carbohydrate. Little attention however has been devoted to dietary

proteins. This is somewhat surprising since dietary proteins can influence serum lipids, especially cholesterol.

In 1958 Olson et al. (40) observed a decrease in serum cholesterol in human subjects when their daily protein intake was changed from 100 grams animal protein to 25 grams of vegetable protein derived from cereals, rice and legumes. The following year Kritchevsky and coworkers (41) also noted the hypercholesterolemic effect of dietary animal protein. Chickens were fed a diet which contained either casein or Promine (a soybean protein) as the protein source. The chickens fed casein had a serum cholesterol of 539 mg/dl, whereas, those on Promine had levels of 368 mg/dl. In 1976 Hamilton (42) compared the effects of dietary animal and vegetable proteins on serum cholesterol levels in rabbits. Animal proteins were again demonstrated as being more hypercholesterolemic when compared to vegetable proteins. The various animal proteins tested produced serum cholesterols ranging from 105 to 235 mg/dl, whereas, vegetable protein levels ranged from 15 to 80 mg/dl. Additional data have confirmed that dietary vegetable proteins when compared to animal proteins are hypocholesterolemic (43,44).

In 1960 Walker et al. (45) studied the effect of dietary vegetable protein versus animal protein on the concentration of cholesterol in the serum of young women.

They concluded that vegetable protein was hypocholesterolemic when compared to animal protein as evidenced by the mean serum cholesterol for animal and vegetable protein being 157 mg/dl and 137 mg/dl respectively after only six weeks of feeding. Hodges et al. (46) also demonstrated that hypercholesterolemia could be reduced by substituting dietary vegetable protein for animal protein. A recent study on the effect of dietary protein on serum lipids was conducted by Sirtori et al. (47). The substitution of vegetable proteins for animal proteins produced a 32 per cent decrease in serum cholesterol and a 16 per cent decrease in serum triglycerides.

Several researchers have not observed a lowering of serum cholesterol when dietary vegetable proteins were substituted for animal proteins. Anderson, Grande and Keys (48) reported no significant difference in serum cholesterol or triglyceride levels in men when comparing the dietary proteins egg albumin and gluten. Recently, Nerves et al. (49) compared the effects of various dietary animal and vegetable proteins on serum lipids in rats and concluded that vegetable proteins were not hypocholesterolemic when compared to animal proteins.

Recognizing that dietary protein may influence serum lipids, Kritchevsky and Klurfeld (50) recently demonstrated that dietary vegetable protein markedly reduced the formation

of cholesterol gallstones in hamsters. Fifty-eight per cent of the hamsters contracted gallstones when fed a diet containing casein as compared to 14 per cent of the hamsters on a soybean diet. Furthermore, once cholesterol gallstones were present in the hamster, dissolution of the stone was accomplished with the administration of the soybean protein diet.

There is clear evidence that diet has an effect on cholelithiasis although the mechanisms are not clearly defined. The relationship between dietary protein and cholelithiasis is one of particular interest since protein derived from animal sources has a greater tendency to elevate plasma cholesterol than protein derived from plants. Physiological effects of dietary protein need further study in order to ascertain the influence on biliary constituents and cholesterol gallstone formation.

CHAPTER 3

EXPERIMENTAL DESIGN

Selection of Animal Model

Cholesterol gallstones are frequently found in the populations of North America and Europe and can be induced in certain laboratory animals. Many experimental models have been utilized to examine cholesterol cholelithiasis including hamsters, dogs, pigs, sheep and cattle (51). Brenneman et al. (28) stated that the ideal animal model should have the following characteristics: chemical composition of bile should approximate that of human bile, the composition of experimental gallstones should be similar to that of human gallstones, the animal should produce gallstones rapidly with the specific treatment being studied and sufficient quantities of bile and stones should be present for analysis. The hamster meets the above qualifications and therefore, was chosen as the animal model for this study. However, some differences must be noted when comparing gallstone formation in man and the hamster. Gallstone incidence increases with age in man; however, gallstones form more rapidly and to a greater extent in young hamsters. Normally women have a greater risk of developing gallstones; however, male hamsters develop gallstones more frequently

than the female hamster (52).

Description and Care of Animals

Eight-five male hamsters (Mesocricetus auratus, Engle Laboratories, Inc., Farmbersburg, IN) weighing 60 ± 5 g were utilized for this study. The animals were individually housed in metal cages (2½" x 2½" x 1") in a well-ventilated room which was artifically illuminated from 6 AM to 6 PM. Food consumption and body weight for each animal were recorded four times per week.

Upon arrival the animals were fed Purina Rodent Chow (5001), ad libitum, for 7 days. After the equilibration period the animals were randomly assigned to one of four experimental groups or to the control group and fed their respective diets (Table 1,2,3, and 4) for 45 days. Diets and water were provided ad libitum throughout the experiment.

This study utilized the Dam Diet to compare the effects of dietary animal and vegetable proteins on cholesterol cholelithiasis and biliary constituents. The success of the gallstone inducing Dam Diet relies upon a high sucrose composition, 74.3 per cent. Protein comprises 20 per cent of the diet with the balance being minerals and vitamins. In order to obtain a diet this high in sucrose, the protein sources must be pure, containing essentially no carbohydrate, fat or fiber. Pure casein and soybean proteins were obtained for this study. Since cottonseed protein was of particular

TABLE 1

EXPERIMENTAL DIETS

GROUP	COMPOSITION (%)
I*+	74.3% Carbohydrate (Sucrose) 20.0% Protein (Casein) 5.0% Mineral Mix ± 0.5% Vitamin Mix 0.2% Choline Chloride
II	74.3% Carbohydrate (Sucrose) 20.0% Protein (Soybean) 5.0% Mineral Mix ± 0.5% Vitamin Mix 0.2% Choline Chloride
III	74.3% Carbohydrate (64.6% Sucrose + 9.7% from cottonseed flour) 20.0% Protein (Cottonseed) 5.0% Mineral Mix ± 0.5% Vitamin Mix 0.2% Choline Chloride
IV	74.3% Carbohydrate (53.1% Cornstarch + 21.2% Sucrose) 20.0% Protein (Casein) 5.0% Mineral Mix ± 0.5% Vitamin Mix 0.2% Choline Chloride
V	Control - Purina Rodent Chow 5001●

*All diets were obtained from the United States Biochemical Corporation, Cleveland, OH. Cottonseed protein was supplied by Texas A & M University. Composition of cottonseed flour shown in Table 5.

+Dam Diet.

±Mineral and Vitamin composition of diets shown in Tables 2 and 3.

All diets are isocaloric providing approximately 3.5 kcal/g of diet.

●Dietary composition of Purina Rodent Laboratory Chow 5001 is shown in Table 4.

TABLE 2

MINERAL MIXTURE

DIETARY COMPOSITION	PER CENT
$Al_2 (SO_4)_3 \cdot 24 H_2O$	0.009
$CaHPO_4 \cdot 2 H_2O$	11.280
$CaCO_3$	6.860
$Ca (C_6H_5O_7)_2 \cdot 4 H_2O$	30.830
$CuSO_4$	0.008
$Fe (NH_4) (C_6H_5O_7)_2$	1.526
$MgCO_3$	3.520
$MgSO_4$	3.830
$MnSO_4$	0.020
KCl	12.470
KI	0.004
KH_2PO_4	21.880
NaCl	7.710
NaF	0.050
$Cr (C_2H_3O_2)_3$	2×10^{-6}
$ZnCO_3$	5×10^{-5}

TABLE 3

VITAMIN MIXTURE

DIETARY COMPOSITION	g/kg
p-aminobenzoic acid	0.1100
Vitamin C	1.0170
Biotin	0.0004
Ca Pantothenate	0.0660
Choline citrate	3.7150
Folic acid	0.0020
Inositol	0.1100
Vitamin K	0.0500
Nicotinic acid	0.0090
Pyridoxine-HCl	0.0220
Riboflavin	0.0220
Thiamin-HCl	0.0020
Vitamin A (500,000 U/g)	0.0390
Vitamin B_{12}	0.0290
Vitamin D_2 (500,000 U/g)	0.0040
Vitamin E acetate (250 U/g)	0.4850

TABLE 4

PURINA RODENT LABORATORY CHOW 5001

DIETARY COMPOSITION	AMOUNT
Protein (%) - 5-6% animal	23.40
Fat (%)*	4.50
Fiber (%)	5.00
Ash (%)	7.30
Carbohydrate (%)- (no sucrose)	49.80
Calcium (%)	1.20
Magnesium (%)	0.21
Phosphorous (%)	0.80
Potassium (%)	1.10
Sodium (%)	0.40
Chlorine (%)	0.50
Cobalt (mg/kg)	4.00
Copper (mg/kg)	18.00
Fluoride (mg/kg)	35.00
Iodine (mg/kg)	1.70
Iron (mg/kg)	198.00
Manganese (mg/kg)	51.00
Selenuim (mg/kg)	0.004
Zinc (mg/kg)	58.00
Vitamin A (IU/kg)	15000.00
Vitamin D (IU/kg)	5000.00
Biotin (mg/kg)	0.07
Choline (mg/kg)	5000.00
Folic Acid (mg/kg)	5.90
Niacin (mg/kg)	95.00
Pantothenic Acid (mg/kg)	24.00
Riboflavin (mg/kg)	8.00
Thiamin (mg/kg)	17.70
Pyridoxine (mg/kg)	6.00
B-12 (mcg/lb)	10.00
Alpha-tocopherol (IU/lb)	29.80

* Cholesterol content 0.251 to 0.281 mg cholesterol/g of diet.

Digestible energy - 4.25 kcal/g of diet.

interest it was not omitted from this study.

The purest cottonseed flour available contained approximately 60 per cent protein, but also contained 25.04 per cent carbohydrate (Table 5). In order for cottonseed protein to be present at a level of 20.0 per cent of the diet approximately 10 per cent of the diet was complex carbohydrate from the cottonseed flour thus lowering the sucrose concentration to 64.6 per cent as opposed to 74.3 per cent sucrose in the casein and soybean diets. This decrease in the level of sucrose is undesirable since Dam et al.(38,53) have reported that a change or decrease in the level of sucrose or simple carbohydrate decreases the effectiveness of inducing cholesterol cholelithiasis.

In an effort to determine if the aforementioned lowering of the percentage of dietary sucrose would effect cholesterol gallstone formation in the hamster, an additional group was studied. Hamsters in this group were fed a diet containing 74.3 per cent carbohydrate; however, the carbohydrate was comprised of cornstarch and sucrose (5:2 ratio). This diet decreased the level of sucrose to 21.22 per cent with the balance, 53.08 per cent, being cornstarch. This diet was considerably lower in dietary sucrose than was the diet with cottonseed as the protein source. Casein was the protein source at the level of 20.0 per cent.

TABLE 5

COMPOSITION OF COTTONSEED FLOUR *

DIETARY COMPOSITION	PER CENT
Protein	56.56
Carbohydrate	25.04
Fat	3.40
Fiber	2.60
Ash	6.90
Moisture	5.50

* Provided by Texas A & M University.

Free Gossypol - 0.02% or 200,000 ppm.

Collection and Preparation of Samples for Analysis

Prior to sacrifice, animals were fasted for approximately 12 hours receiving only water. Sacrifice was performed between 9 and 11 AM. The hamsters were anesthetized with ether, whereupon the abdomen was opened through a midline incision exposing the liver and the gallbladder. Gallbladder bile was aspirated directly from the gallbladder with a Hamilton syringe. The gallbladder was excised and examined for the presence of gallstones. Hamsters were sacrificed by exsanguination via cardiac puncture with heparinized syringes. The blood samples were immediately centrifuged for 20 minutes at 4,000 rpm at 4°C. Bile and plasma samples were frozen immediately and stored at -20°C for later chemical analysis.

Analytical Techniques

The three major constituents of the gallbladder bile; bile acids, phospholipid and cholesterol, were analyzed quantitatively by the methods of Turley and Dietschy (54), Trudinger (55) and a modification of the Reyes and Kern (56) procedures respectively. Gallbladder bile was used for all bile analyses. Total serum cholesterol was determined quantitatively using the enzymatic method described by Allain et al. (57).

The enzymatic procedure described by Turley and Dietschy (54) was used to assess total bile acids. Bile

acids are oxidized by 3 alpha-Hydroxy-steroid dehydrogenase with the subsequent reduction of Nicotinamide adenine dinucleotide (NAD) to NADH. The amount of NAD reduced to NADH is used to quantitate total bile acid and is analyzed spectrophotometrically at 340 nm.

Biliary phospholipid was analyzed using the method of Trudinger (55). Phosphorous is liberated from biliary phospholipid by digestion with perchloric acid and heat. The liberated free phosphorous reacts with malachite green: ammonium molybdate reagent to form a chromogen which has a maximum absorption of 660 nm.

Gas-liquid chromotography was used to analyze biliary cholesterol (56). Biliary cholesterol was first saponified and the resulting free cholesterol is extracted with chloroform. An aliquot of the organic soluble biliary constituents and known amount of internal standard (stigmasterol) is assayed for biliary cholesterol using gas-liquid chromatography.

The enzymatic method of Allain et al. (57) was used to determine total serum cholesterol. The presence of cholesterol yields a chromogen with a maximum absorption at 500 nm.

The methodology for each analytical procedure utilized is described in detail in the appendix.

Statistical Analysis

The data was collected in a five group single factor design. The basis analysis was a five group analysis of variance, with the error mean square being used as the basis for variability in subsequent tests. There were three comparisions of primary interest: Group I-II, I-III and I-IV. These three comparisons had the highest statistical power while any other comparisons will be made with the less powerful Newman-Keuls multiple range procedure. A value of $P < 0.05$ was considered statistically significant.

IV. RESULTS

The primary objective of this research was to determine the effect of dietary animal and vegetable protein on cholesterol gallstone formation and biliary constituents in the hamster. Additionally total serum cholesterol was examined to determine the effect various dietary proteins have on serum cholesterol and furthermore to determine if there was a correlation between the level of cholesterol in the serum and bile. Finally this study investigated whether the source and/or amount of dietary carbohydrate influences gallstone incidence and biliary constituents.

Weight gain was monitored to establish that changes in biliary metabolism were due to dietary protein alterations and not caused by food rejection. Both of the groups fed casein as a protein source (I and IV) had a significantly lower final weight than did hamsters fed either soybean protein (Group II), cottonseed protein (Group III) or control diet (Group V) (Figure 1).

Effect of Dietary Protein on Cholesterol Gallstone Incidence

A significant decrease in gallstone formation was observed when the source of dietary protein was changed from an animal protein (casein) to a vegetable protein (soybean or cottonseed) (Table 1). Figure 2 illustrates the

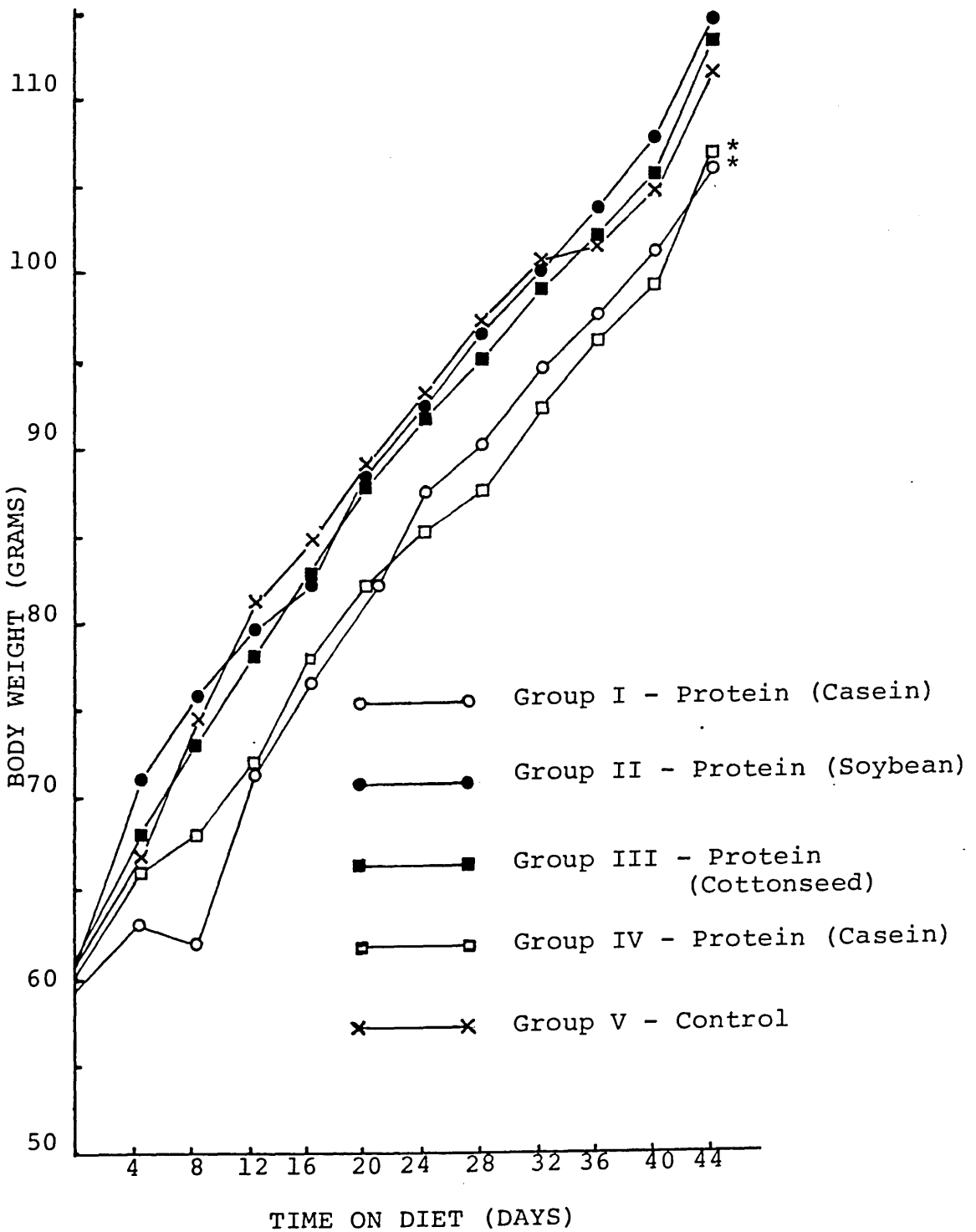

Figure 1. Diet related changes in body weight of Hamsters as related to length of time on on diet.

*Significantly lower at ($P<0.05$).

TABLE 6

EFFECT OF DIETARY PROTEIN ON CHOLESTEROL GALLSTONE INCIDENCE

GROUP	PROTEIN SOURCE	SURVIVAL	GALLSTONES (%)
I	CASEIN	17/18	100.0
II	SOYBEAN	19/19	31.6
III	COTTONSEED	16/16	0.0
V	CONTROL	17/17	0.0

cholesterol gallstones present in the gallbladder of hamsters fed casein. No gallstones were present in the gallbladder or biliary duct of the control animals.

Effect of Dietary Protein on Biliary Constituents

In an effort to ascertain the mechanism or mechanisms responsible for gallstone formation with respect to the experimental diets, the bile was quantitatively analyzed for the three major biliary constituents; bile acids, phospholipid and cholesterol. These three constituents are reported in both absolute (μmol/ml) and relative (molar %) concentration (Table 2). The primary interest is the possible differential effect of animal and vegetable proteins with respect to the concentration of biliary bile acids, phospholipid and cholesterol. Of ancillary interest is a comparison between two experimental groups (I-74.3% sucrose and IV-53.1% cornstarch and 21.2% sucrose) which will focus on the influence of the complexity of dietary carbohydrate on gallstone formation and biliary constituents.

Effects on Bile Acid Concentration

No significant difference was observed between the absolute concentration of bile acids. Mean absolute concentration of bile acids was significantly higher in the control group than in any experimental group. The relative concentration of bile acids in gallbladder bile

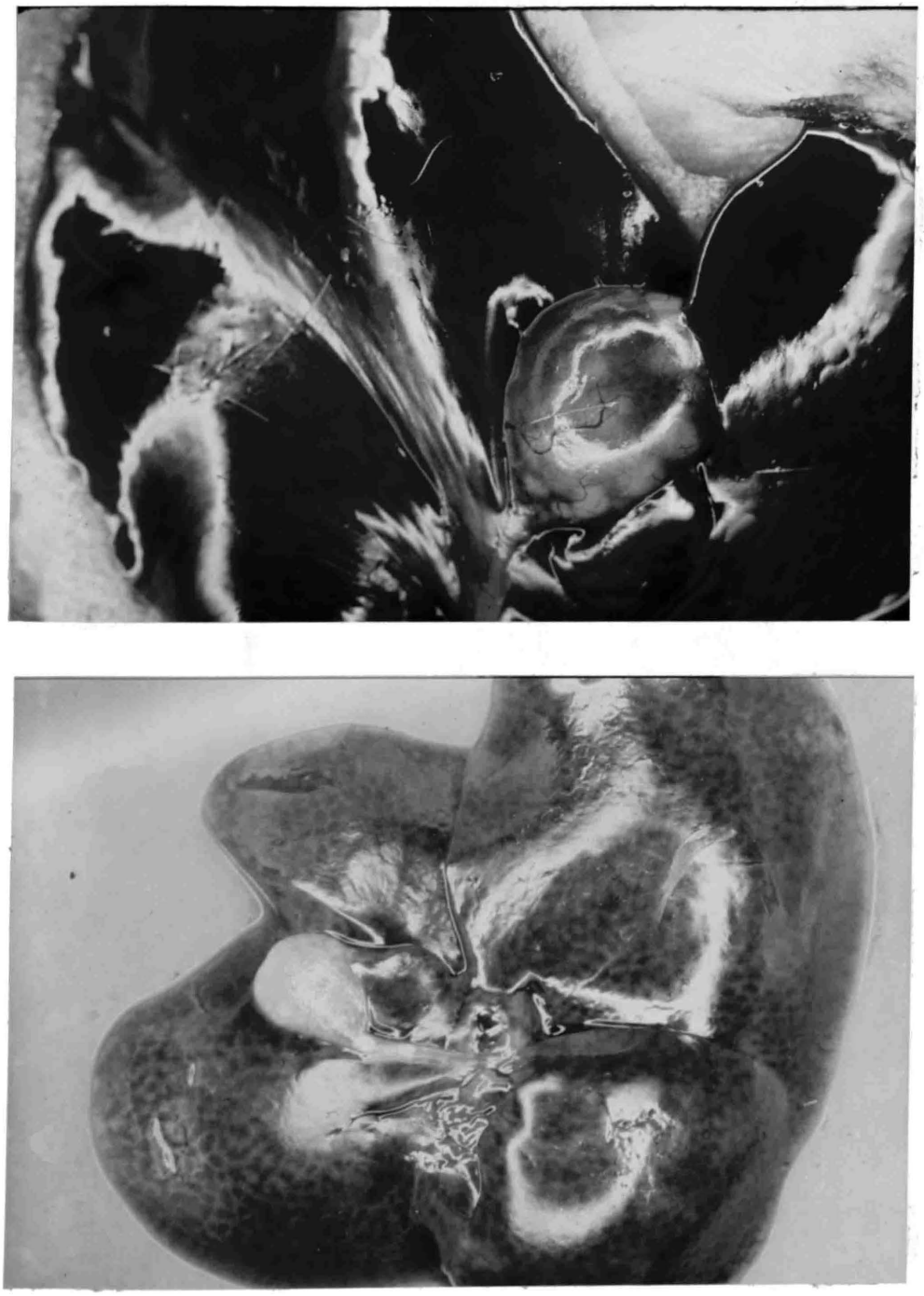

Fig. 2 Cholesterol gallstones in the gallbladder of hamsters fed casein protein (Group I).

TABLE 7

EFFECT OF DIETARY PROTEIN ON BILIARY COMPOSITION OF HAMSTERS

PROTEIN SOURCE	N	ABSOLUTE CONCENTRATION (μmol/ml)			RELATIVE CONCENTRATION (molar %)		
		BILE ACID	PHOSPHOLIPID	CHOLESTEROL	BILE ACID	PHOSPHOLIPID	CHOLESTEROL
CASEIN	9	140.0 ± 15.4[a]*	17.4 ± 2.3[a]	6.30 ± 0.52[a]	85.2 ± 0.71[a]	10.51 ± 0.41[a]	4.31 ± 0.67[a]
SOYBEAN	18	140.9 ± 7.3[a]	17.4 ± 1.4[a]	5.18 ± 0.35[b]	86.4 ± 0.49[a]	10.44 ± 0.45[a]	3.16 ± 0.14[a]
COTTONSEED	15	116.9 ± 7.6 [a]	12.0 ± 1.3[b]	3.03 ± 0.26[c]	88.9 ± 0.54[b]	8.81 ± 0.51[b]	2.27 ± 0.09[b]
CONTROL	17	223.8 ± 11.7[b]	16.7 ± 1.3[a]	2.74 ± 0.26[c]	92.1 ± 0.33[b]	6.77 ± 0.29[c]	1.11 ± 0.08[c]

*Mean±SE. Means in the same column bearing different superscript letters are significantly different ($P<0.05$).

of animals fed casein or soybean as the protein source did not differ significantly; however, the percentage of bile acids in hamsters fed cottonseed was significantly greater than with either of the above. The control group had a significantly higher relative concentration of bile acids than any of the experimental groups.

Effects on Biliary Phospholipid Concentration

The absolute concentration of phospholipid present in bile from hamsters fed cottonseed protein were significantly lower than from animals fed casein, soybean protein or laboratory chow while no differences were noted among the latter three groups. The relative concentration of biliary phospholipid of animals fed cottonseed protein was significantly lower than that of animals fed casein or soybean protein with no difference between the latter two groups. The relative concentration of biliary phospholipid was significantly lower in the control animals when compared to any experimental group.

Effects on Biliary Cholesterol

When casein was the protein source, hamsters had a significantly higher absolute concentration of biliary cholesterol than did animals fed either of the two vegetable proteins or the control diet. The relative concentration of cholesterol in the bile of hamsters fed casein was higher

than any of the other groups. Casein fed animals had a higher percentage of biliary cholesterol than did animals fed soybean protein; however, this difference was not statistically significant. Animals fed cottonseed protein had a significantly lower percentage of biliary cholesterol when compared to either casein or soybean protein fed animals. The percentage of biliary cholesterol was significantly lower in the control group when compared to either casein or soybean fed animals.

Effect of Dietary Protein on Total Serum Cholesterol

Serum cholesterol was lowered when casein was replaced with soybean; however, this difference was not significant (Table 3). The replacement of casein with the vegetable protein from cottonseed significantly lowered total serum cholesterol. All of the experimental diets had significantly higher total serum cholesterol than did the animals fed laboratory chow.

This research indicates a moderate correlation (Correlation cofficient 0.547) when comparing the level of cholesterol in the serum and bile (Table 4). The animals fed casein as the protein source produced the highest levels of serum and biliary cholesterol as well as the greatest incidence of cholesterol gallstone formation. Cottonseed protein produced significantly lower levels

TABLE 8

EFFECT OF DIETARY PROTEIN ON TOTAL SERUM CHOLESTEROL

GROUP	PROTEIN SOURCE	SERUM CHOLESTEROL (mg/dl)
I	CASEIN	212.60 ± 10.81^{a}
II	SOYBEAN	198.24 ± 5.08^{ab}
III	COTTONSEED	180.61 ± 8.69^{b}
IV	CASEIN (CORNSTARCH:SUCROSE)	248.52 ± 11.87^{c}
V	CONTROL	122.86 ± 7.97^{d}

Means $\pm$ SE. Means in the same column bearing different superscript letters are significantly different ($P<0.05$).

TABLE 9

EFFECT OF DIETARY PROTEIN ON CHOLESTEROL CONCENTRATION OF GALLBLADDER BILE AND SERUM

PROTEIN	N	GALLSTONES (%)	GALLBLADDER BILE (μmol/ml)	SERUM (mg/dl)
CASEIN	16	100.0	6.30 ± 0.52^{a}	212.60 ± 10.81^{a}
SOYBEAN	19	31.6	5.18 ± 0.35^{a}	198.24 ± 5.08^{ab}
COTTONSEED	16	0.0	3.03 ± 0.26^{b}	180.61 ± 8.69^{b}
CONTROL	17	0.0	2.74 ± 0.26^{a}	122.86 ± 7.97^{d}

Means ± SE. Means in the same column bearing different superscript letters are significantly different ($P < 0.05$).

of serum and biliary cholesterol than casein fed hamsters and produced a significant decrease in gallstone incidence.

Effect of Dietary Carbohydrate on Cholesterol Gallstone Formation and Biliary Constituents

The effect of dietary carbohydrate on cholesterol gallstone formation and biliary constituents in the hamster is shown in Table 5. Group I was fed a diet containing 74.3 per cent sucrose while Group IV was fed a diet that also contained 74.3 per cent carbohydrate; however, the carbohydrate was composed of cornstarch and sucrose (5:2 ratio). No difference was observed in the absolute concentration of bile acids or phospholipid when comparing the two groups; although, the level of biliary cholesterol was significantly higher in Group I (6.30 μmol/ml) when compared to Group IV (5.12 μmol/ml). The relative concentration of bile acids was significantly higher in animals fed cornstarch:sucrose mixture when compared to animals fed sucrose alone. The relative concentration of biliary cholesterol was increased in animals fed sucrose versus animals fed a mixture of cornstarch and sucrose; however, this difference was not statistically significant.

TABLE 10

EFFECT OF DIETARY CARBOHYDRATE ON CHOLESTEROL GALLSTONE FORMATION AND BILIARY CONSTITUENTS

BILIARY CONSTITUENT	DIET I (n=9)	DIET IV (n=17)
I. ABSOLUTE CONCENTRATION: (μmol/ml)		
BILE ACIDS	140.0 $\pm$ 15.4 [a]	136.6 $\pm$ 5.1 [a]
PHOSPHOLIPID	17.4 $\pm$ 2.3 [a]	15.8 $\pm$ 1.0 [a]
CHOLESTEROL	6.30 $\pm$ 0.52 [a]	5.12 $\pm$ 0.39 [b]
II. RELATIVE CONCENTRATION:		
BILE ACIDS	85.2 $\pm$ 0.71 [a]	86.8 $\pm$ 0.38 [b]
PHOSPHOLIPID	10.51 $\pm$ 0.41 [a]	9.96 $\pm$ 0.31 [a]
CHOLESTEROL	4.31 $\pm$ 0.67 [a]	3.24 $\pm$ 0.21 [a]
GALLSTONE FORMATION (%)	100.0	38.9

Mean $\pm$ SE. Means in the same column bearing different superscript letters are significantly different ($P<0.05$).

IV. SUMMARY AND CONCLUSIONS

Gallstone incidence was dramatically reduced in the hamster when a vegetable protein (soybean or cottonseed) was substituted for an animal protein (casein). When casein was utilized as the protein source gallstones were produced in 100 per cent of the animals; however, with the substitution of soybean or cottonseed as the protein source, gallstone incidence was significantly reduced to 32 per cent and 0 per cent respectively. Similar observations were reported by Kritchevsky and Klurfeld (50) with dietary animal protein (casein) being more lithogenic than vegetable protein (soybean). Fifty-eight per cent of the hamsters fed casein developed cholesterol gallstones compared to 14 per cent gallstone incidence in hamsters fed soybean.

The absolute concentration of biliary cholesterol was increased in all experimental diets when compared to control animals. With casein, soybean and cottonseed producing mean biliary cholesterol levels of 6.30, 5.18 and 3.03 μmol/ml respectively. Casein fed hamsters had a significantly higher absolute concentration on biliary cholesterol than did hamsters fed the vegetable proteins (soybean or cottonseed). Hamsters fed soybean produced a significantly greater

absolute concentration of biliary cholesterol than did animals receiving cottonseed as the protein source. The relative concentration of biliary cholesterol of hamsters fed casein was higher than in all other groups. Kajiyama et al. (25) reported similar values for biliary cholesterol (molar %) when using the Dam Diet with casein as the protein source. Casein fed animals had a higher percentage of biliary cholesterol than did animals fed soybean; however, this difference was not significant. It appears that this lack of difference is directly effected by the large standard error of the mean with respect to the relative concentration of cholesterol of casein fed animals. When cottonseed was the protein source, biliary cholesterol was significantly lower when compared to either casein or soybean.

Total serum cholesterol was quantitatively measured to determine if the type of dietary protein, animal or vegetable, would influence the level of cholesterol in the serum. Serum cholesterol was lowered when soybean replaced casein as the protein source; however, this difference was not significant. The replacement of casein with the vegetable protein cottonseed did significantly decrease total serum cholesterol. This research has supported earlier observations that vegetable proteins are more hypocholesterolemic when compared to animal

proteins in both animals and man (40-47).

This study indicates that there does appear to be a positive correlation between the level of cholesterol in the serum and gallbladder bile. Casein fed animals had the highest mean absolute concentration of biliary cholesterol, 6.30 μmol/ml $\pm$ 0.52, and the highest mean serum cholesterol 212.60 mg/dl $\pm$ 10.81 when compared to diets where soybean and cottonseed were the protein sources. Conversely the animals fed cottonseed as the protein source had the lowest absolute concentrations of biliary cholesterol, serum cholesterol and gallstone incidence.

As previously stated the type of carbohydrate utilized in the Dam Diet has been reported to effect gallstone formation. Dam et al.(38) demonstrated that the replacement of sucrose with complex carbohydrates markedly inhibited cholesterol gallstone formation. This research indicates that the type of dietary carbohydrate does influence gallstone incidence when using the gallstone inducing Dam diet. One hundred per cent of the hamsters developed gallstones when fed the Dam diet (Group I) as compared with 38.9 per cent of the hamsters who formed gallstones when the amount of sucrose in the diet was reduced to 21.2 per cent with the balance of carbohydrate consisting of 53.1 per cent cornstarch (Group IV). When the level of sucrose was reduced to 21.2 per cent (Group IV) of the diet, gallstone

incidence was moderately reduced to 38.9 per cent. The diet containing cottonseed as the protein source which produced a dramatic decrease in gallstone incidence does not appear to be solely attributable to a decrease in dietary sucrose. This research indicates that when cottonseed was used as the protein source it appears to be partially responsible for the significant decrease in gallstone formation.

The proposed mechanism which resulted in gallstone formation in Group I versus Group IV was a higher absolute and relative concentration of biliary cholesterol.

After reviewing the data from this research the following conclusions are offered:

1. The substitution of vegetable for animal protein in the diet can dramatically decrease gallstone formation in hamsters.
2. A lowering of the concentration of biliary cholesterol in an absolute and relative manner appears to be partially responsible for the decrease in gallstone incidence.
3. Animal protein (casein) was hypercholesterolemic when compared to the vegetable protein soybean or cottonseed.

4. A positive correlation between the level of cholesterol in the serum and gallbladder bile was noted which suggests that factors which cause a rise in serum cholesterol may also influence the concentration of biliary cholesterol.

The consideration of the aforementioned information suggests that a reduction in the amount of animal protein in the diet with the substitution of vegetable proteins could offer an alternative treatment for cholesterol cholelithiasis.

More research is needed concerning the comparative effects of dietary protein on cholesterol gallstone formation and biliary lipid composition to determine if all dietary animal proteins are lithogenic when compared to vegetable proteins. Additionally dietary proteins should be studied to determine if a decrease in biliary saturation with the subsequent dissolution of cholesterol cholelithiasis is possible with dietary intervention.

METHOD FOR DETERMINING TOTAL BILE ACID (54)

Principle

Bile acids are oxidized by 3 alpha-Hydroxysteroid dehydrogenase (3α-HSD) with the subsequent reduction of Nicotinamide adenine dinucleotide (NAD) producing NADH. To insure complete oxidation of the bile acids, hydrazine hydrate is used as a ketone-trapping agent. The amount of NAD reduced to NADH is used to quantitatively measure the total bile acid.

Reagents

1. Tris-HCl Buffer pH 9.5:
   a. Tris (0.133 M) 16.11 g
   b. EDTA, disodium salt (0.666 mM) 223.0 mg
   Add Tris and EDTA, disodium salt to 1000 ml distilled water. Adjust pH to 9.5 with the addition of concentrated HCl. Stable for 3 months at room temperature.
2. Tris-HCl Buffer pH 7.2:
   a. Tris (0.03 M) 3.64 g
   b. EDTA, disodium salt (1mM) 336.2 mg
   Add Tris and EDTA, disodium salt to 1000 ml of distilled water. Adjust pH to 7.2 with the addition of

concentrated HCl. Stable 3 months at room temperature.

3. Hydrazine hydrate (1M) pH 9.5:

   a. Hydrazine hydrate (100%)

   b. 2 N H_2SO_4

   c. Distilled water

   Under a hood add 50 ml hydrazine hydrate to approximately 200 ml distilled water and chill in ice. Add 15 ml 2N H_2SO_4 slowly and then make up to 1000 ml with distilled water. This gives a 1 M solution, pH 9.5. Stable 3 months in dark bottle at room temperature.

4. NAD 7 mM, pH 7.0:

   a. β-NAD (Sigma Chemical Co. - Grade III - #N-7004)

   b. Sodium Bicarbonate

   Dissolve 464.5 mg NAD in distilled water and make to a final volume of 100 ml. Adjust pH to 7.0 by adding solid sodium bicarbonate. Stable 2 weeks when stored at $4^{o}C$.

5. Bile Salt Standard:

   a. Sodium Taurocholate (Calbiochemical-Behring Corp.-Grade A #580217)

   b. Methanol

   2.0 mM- 107.5 mg sodium taurocholate in a final

volume of 100.0 ml methanol. Concentration - 0.2 μmol bile acid/0.1 ml. Stable 4 months when stored at 4°C.

6. 3α-HSD:

   a. 3α-HSD (Worthington, STDHMP code - #LSOO 04910)

   b. Tris-HCl Buffer pH 7.2 (Cold)

   Prepare enzyme at a concentration of 2 units of enzyme/ml buffer. Stable one week when stored at 4°C.

Analysis of Bile

Prepare all samples in disposable glass culture tubes (12 x 75 mm).

1. Premix Tris-HCl Buffer pH 9.5, Hydrazine hydrate, and NAD in the correct volume for all tubes required.

   EXAMPLE: Reaction Mixture for Bile Acid Assay

# of Tubes	Total Vol. (ml)	Tris HCl Buffer pH 9.5 (ml)	Hydrazine Hydrate (ml)	NAD (ml)
1	2.8	1.5	1.0	0.3
50	140.0	75.0	50.0	15.0
100	280.0	150.0	100.0	30.0

2. Prepare the following tubes:

   Sample: (Prepare in duplicate)

   a. 2.8 ml reaction mixture

   b. 1.0 μl bile

   c. 0.1 ml methanol

   d. 0.1 ml 3α-HSD

Sample Blank: A sample blank is sometimes required if bile

contains high amounts of bile pigments which would falsely elevate the optical denisty (O.D.) of the sample; however this is unusual.

a. 2.8 ml reaction mixture

b. 1.0 µl bile

c. 0.1 ml methanol

d. 0.1 ml Tris-HCl Buffer pH 7.2

Standard: (Prepare in triplicate)

a. 2.8 ml reaction mixture

b. 0.1 ml sodium taurocholate standard = 0.2µmol bile acid

c. 0.1 ml 3α- HSD

Standard Blank: (Prepare in triplicate)

a. 2.8 ml reaction mixture

b. 0.1 ml methanol

c. 0.1 ml 3α-HSD

Vortex all reagents before adding enzyme. Vortex gently after addition of enzyme to insure mixing.

3. Incubate all tubes in water bath at 30°C for 1 hour and then read at 340 nm. Zero the spectrophotometer with distilled water. The ΔOD of the sample and the standard is obtained by subtracting either the O.D. of the respective sample blank or the reagent blank, whichever is higher.

Calculation

1. O.D. of STD - O.D. of Reagent Blank = O.D. for STD
2. O.D. of Sample - O.D. of Reagent Blank = Δ O.D. for Sample

$$\frac{\text{Conc. of STD } (\mu\text{mol})}{\Delta \text{ O.D. of STD}} \times \Delta \text{ O.D. Sample} = \frac{\mu\text{mol Bile Acid}}{\mu\text{l Bile}}$$

μmol Bile Acid/μl Bile x 1000 = μmol Bile Acid/ml Bile

METHOD FOR DETERMINING BILIARY PHOSPHOLIPID (55)

Principle

Phosphorous is liberated from the phospholipid by digestion with perchloric acid and heat. The liberated phosphorous reacts with malachite green:ammonium molybdate reagent to form a chromogen that has a maximum absorption at 660 nm.

Reagents

Use Glass Distilled Water (GDW) for all agueous solutions.

1. 70% Perchloric Acid.
2. 4.2% Ammonium Molybdate in 5N HCl:
   a. 5 N HCl - Add 415 ml concentrated HCl to 585 ml GDW.
   b. Dissolve 42 g Ammonium Molybdate in 1000 ml 5N HCl.
3. 0.2% Malachite Green:
   Dissolve 4 g malachite green in 2000 ml GDW.
4. No. 2 Whatman Filter Paper.
5. 1.5% Tween 20.
6. Standards:
   a. Inorganic Standard - K_2HPO_4 - Dissolve 174.8 mg K_2HPO_4 in 100 ml GDW (Final Volume). Dilute this 1:100 with GDW to give working standard at a concentration of 1 nmol/10 μl. Store at 4°C.

b. Organic Standard - Diphenylphosphinic Acid (Aldrich Chemical Comp. - 99% purity - #10,852-9). Dissolve 21.81 mg diphenylphosphinic acid in 100 ml ethanol (final volume). Dilute this 1:10 with ethanol to give working standard at a concentration of 1 nmol/10 μl.

Analysis of Sample

Prepare all samples in disposable glass culture tubes 12 x 75 mm. The disposable tubes must be rinsed three times with GDW to prevent phosphorous contamination.

1. Add 1 μl bile to culture tubes, prepare samples in triplicate.
2. Take triplicate 50,100, and 200 μl aliquots of both the inorganic and organic standards yielding 5, 10, and 20 nmol of phosphorous (P) respectively. Evaporate tubes to dryness under stream of nitrogen gas.
3. Add three empty tubes for blanks.
4. Add 50 μl 70% perchloric acid to all tubes and vortex. Place tubes in sand bath ($200^{o}C \pm 20^{o}C$) under a hood and digest for 90 minutes.
5. During digestion process, prepare the following reagent: Mix 3 parts 0.2% malachite green with one part 4.2% ammonium molybdate reagent. Stir for 30 minutes. Filter malachite green:ammonium molybdate (MG:AM) mixture twice

through Whatman filter paper.

6. After 90 minutes in sand bath, remove tubes and let them cool.
7. Add 400 µl GDW and vortex.
8. Add 2 ml MG:AM solution followed immediately by 100 µl of 1.5% Tween and vortex.
9. Read at 660 nm between 30 to 60 minutes later. Zero the spectophotometer with blanks.

Calculation

$$\text{nmol P/ µl bile} = \frac{\text{Conc. std.(nmol)*}}{\text{O.D. std. (nm)}} \times \text{O.D. sample}$$

$$\text{mo P/ ml bile} - \text{nmol P/ µl bile} \times \frac{1000}{1000}$$

*The average concentration of the three standards; 5, 10, and 20 nmol, is 11.667 nmol of P.Average the respective O.D.'s for the standards to find the O.D. = to 11.667 nmol of phosphorous.

METHOD FOR DETERMINING BILIARY CHOLESTEROL (56)

Principle

Biliary cholesterol esters are first saponified by heating with alkali. The resulting free cholesterol is extracted by the addition of chloroform. The organic soluble biliary constituents and a known amount of stigmasterol (internal standard) are quantitatively measured using gas-liquid chromatography.

Reagents

1. Stigmasterol (Applied Science Division - #19560): Dissolve 10.00 mg stigmasterol in 100 ml absolute ethanol (final volume . Concentration 1 μg stigmasterol/10 μl absolute ethanol. Store at $4^{o}C$.
2. Ethanolic - Potassium Hydroxide (ETOH-KOH):
   a. Absolute ethanol
   b. 50% Potassium Hydroxide

   Gradually add 3 ml of 50% KOH to 47 ml absolute ethanol.
3. 40% Ethanol-40 ml absolute ethanol + 60 ml distilled water.
4. Chloroform - Burdick and Jackson Solvents - Distilled in Glass.
5. Heptane - Burdick and Jackson Solvents - Distilled in Glass.
6. Glass Column - U shaped 3 ft x 2 mm interior diameter

(I.D.) Supelco Inc. - #2-0610).

7. Packing - 3% SP-2250 on 100-120 Supelco Support (Supelco, Inc. - #1-1878).

Sample Preparation

Prepare all samples in disposable glass culture tubes (12 x 75 mm).

1. Add 50 μl stigmasterol standard (contains 5 μg stigmasterol).
2. Add 500 μl ETOH-KOH mixture.
3. Add 5 μl bile.
4. Vortex
5. Heat the tubes at 55-60^{o}C in water bath for one hour. Vortex tubes three times during this one hour period.
6. To the cooled tubes, add 2 ml chloroform and 1 ml of 40% ethanol and vortex.
7. Centrifuge the tubes at 1500 rpm for 5 minutes. Cold centrifugation results in better separation. The mixture will have separated into 2 layers or phases, the top layer is water (40% ETOH) and the bottom layer is chloroform.
8. Aspirate the water layer off as completely as possible leaving the 2 ml of chloroform.
9. Add 1 ml of 40% ETOH, vortex, centrifuge at 1500 RPM for 5 minutes and aspirate water layer leaving the 2 ml of chloroform.

10. Repeat steps #9 two more times.
11. Evaporate the chloroform layer to dryness under stream of nitrogen gas. Place the tubes over a warm water bath (40°C) while evaporating the chloroform to speed the evaporation process. Seal dried tubes.
12. At time of injection, add 15 μl of heptane to dried tubes and vortex. Inject 3 μl of the total 15 μl.

Analysis of Sample

Column Conditions:

1. Oven Temperature - Initial 250°C. Must increase oven temperature to 300°C after stigmasterol peak to insure evaporation of bile acid peak.
2. Inlet or Injector Temperature - 260°C.
3. Detector (Flame Ionization Detector) - 350°C
4. Transfer Temperature - 340°C
5. Carrier Gas Flow Rate - 23 ml Nitrogen gas/minute
6. Retention Times:

   Cholesterol - 7.5 to 8.5 minutes.

   Stigmasterol - 10.8 to 11.5 minutes.

   Bile Acids - 19.0 to 20.5 minutes.

Calculation

Cholesterol - C Stigmasterol - S

Correction Factor for Gas Chromatograph - C_f

$$\mu g \text{ Cholesterol}/\mu l \text{ Bile} = \frac{\text{area C}}{\text{area S}} (x \text{ or } \div C_f) \; x \mu g \text{ Stigmasterol}$$

METHOD FOR DETERMINING TOTAL

SERUM CHOLESTEROL (57)

Principle

Cholesterol esters are hydrolyzed to free cholesterol by cholesterol esterase. The free cholesterol produced is oxidized by cholesterol oxidase simulatenously producing cholest-4-en-3-one and hydrogen peroxide with 4-amino-antipyrine and phenol yielding a chromogen with maximum absorption at 500 nm.

Reagents

1. Reagent A (for 100 Assays): To 300 ml distilled water add: 390.0 mg sodium cholate; 50.0 mg 4-aminoantipyrine; 400.0 mg. phenol; 400.0 mg carbowax; 4475.2 mg $NaHPO_4$, sodium phosphate, monobasic; 2300.0 mg NaH_2PO_4, sodium phosphate, dibasic. Vortex reagents until well mixed. Add 10.0 units cholesterol esterase (Sigma Chemical Co. - #C-1892); 40.0 units cholesterol oxidase (Sigma Chemical Co.); and 20100.0 units cholesterol peroxidase (Miles Biochemicals - #36-451-1). Mix enzymes into above reagents gently with glass stirring rod. Reagent stable for 3 to 4 days when stored in dark bottle at $4^{o}C$ or 8 hours at room temperature.
2. Triton X-100

3. 0.90% Saline

4. Triton X-100/Saline (1:100 v/v)

5. Cholesterol Stock Standard for serum: Dissolve 300 mg. cholesterol in 10.0 ml Triton X-100. Dilute to 100 ml with 0.90% Saline.

6. Cholesterol Working Standard:

Cholesterol (mg/dl)	Stock Std. (ml)	Triton X-100/ Saline
50	0.5	2.5
100	1.0	2.0
200	2.0	1.0
300	3.0	0.0

Analysis of Serum or Plasma

1. Prepare the following tubes:

   a. Blank: 3.0 ml Reagent A

   b. Standard: 3.0 ml Reagent A + 25 µl Working Std.

   c. Standard Blank: 3.0 ml Reagent A + 25 µl Triton X-100/ Saline

   d. Sample: 3.0 ml Reagent A + 25 µl Serum *

   e. Sample Blank: 3.0 ml 0.90% Saline + 25 µl Serum *

   Gentle Vortex tubes to insure mixing.

2. Incubate all tubes at 37°C for 30 minutes. Immediately read optical denisty (O.D.) against the blank at 500 nm. Subtract O.D. of standard and sample blanks from the O.D. of standard and sample respectively.

* Serum/Plasma

Calculation

$$\text{mg cholesterol/dl serum} = \text{O.D. sample} \times \frac{\text{conc. std (mg/dl)}}{\text{O.D. standard}}$$

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