THE EFFECT OF DIETARY VEGETABLE AND ANIMAL PROTEIN ON THE ETIOLOGY OF CHOLESTEROL GALLSTONE FORMATION IN THE HAMSTER

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We hereby recommend that the thesis prepared under our supervision by Annemarie Strobl Richmond entitled The Effect of Dietary Vegetable and Animal Protein on the Etiology of Cholesterol Gallstone Formation in the Hamster

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D E D I C A T I O N

This thesis is dedicated to the memory

of my parents

Professor and Mrs. Gustav Strobl

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CHAPTER 1

INTRODUCTION

Cholesterol gallstone disease, cholelithiasis, is a painful, widespread and costly disease in affluent Western societies. The etiology of this disease appears to lie in a combination of factors which relate genetic and environmental effects to endocrinological and nutritional abnormalities. Portions of the mechanism have been demonstrated to affect gallstone disease in humans and research animals.

The formation of cholesterol gallstones has been associated with abnormalities in the relative concentration of the three bile constituents: cholesterol, bile acid (BA), and phospholipid (PL) (1). Gallstone formation proceeds in three stages: Bile becomes saturated or supersaturated with cholesterol, nucleation occurs, followed by growth of the precipitated crystals to symptomatic size (2).

Supersaturation of bile with cholesterol may be increased by a number of factors. The cholesterol concentration in bile may be increased by high rates of cholesterol secretion from the liver or reduced secretion of BAs and PLs. The secretion of hepatic cholesterol into

bile may be stimulated through increased cholesterogenesis due to an increase of the rate-limiting enzyme 3-hydroxy-3-methylglutaryl Coenzyme A reductase (EC 1.1.1.34) (3). Reduced conversion of cholesterol to BAs, mobilization of cholesterol from tissue pools, and increased ingestion and absorption of cholesterol are important factors which may also affect cholesterol secretion (2).

The amount of cholesterol that can be kept in solution in the bile depends on the relative concentration of bile salts and PLs in the bile. In normal bile the ratio of cholesterol to bile salts is 1:20-1:30. If this ratio falls to 1:13, precipitation of cholesterol crystals can occur (4).

Insufficient concentration of BAs may result from an accelerated loss of BAs from the body which exceeds the ability of the normal liver to compensate with increased BA synthesis. Faulty regulation of BA synthesis through enterohepatic circulation or an inability of the liver to secrete adequate amounts of BAs are other causes for diminished BA concentration. These factors may lead to correspondingly higher cholesterol concentrations in the bile (2, 5, 6, 7).

Decreased synthesis and secretion of PLs could also contribute to enhanced saturation of bile with cholesterol.

Lecithin can suppress cholesterol absorption by either having a direct effect on the mucosal cell membrane, or by holding cholesterol in a micellar form in the lumen of the intestine (8).

The complete mechanism behind bile cholesterol supersaturation and concomitant changes in the relative concentration of BAs and PLs is not clearly understood. Some controversy exists as to whether an increase in serum lipid concentration leads to an increased incidence of cholesterol gallstone disease. Very early epidemiological and clinical data suggest an increased risk for gallbladder disease in patients with coronary heart disease (9, 10). Swedish researchers have found that male and female patients with gallstone disease have higher serum triglyceride concentrations when compared with controls. Serum cholesterol concentrations were not found to be linked positively with conditions leading to the formation of gallstones (11-15). The report from this research group could not be confirmed in a similar study in Scotland, where gallstone disease did not appear to be associated with abnormal serum lipid concentrations or ischemic heart disease (16).

Serum cholesterol lipid fractions have been found to constitute a reliable predictor of atherosclerosis. As concentrations of low density lipoproteins (LDL) in serum

rise, and high density lipoproteins (HDL) decrease, the incidence of coronary heart disease (CHD) increases (17). Very low density lipoproteins (VLDL) and LDL are synthesized in the small intestine and in the liver and carry cholesterol and triglycerides to the visceral and peripheral organs for cellular use or storage. Serum HDL bind peripheral cholesterol and prevent its uptake by smooth muscle and other extrahepatic tissues. High density lipoproteins then transport cholesterol to the liver for synthesis of BAs and/or other sterol compounds. Through this mechanism, HDL aids in the removal of cholesterol from serum, thus preventing excessive tissue storage and an associated high risk of atherosclerosis (18). Little is known about how changes in serum lipid concentrations may be associated with changes in biliary lipids. The manner in which the distribution of serum LPs affects gallstone formation is not well understood. Cholesterol which is returned to the liver by HDL is used primarily for BA synthesis and excretion into bile (19). One may speculate, therefore, that higher serum HDL levels may lead to increased BA synthesis in the liver and higher BA concentration in the gallbladder.

Intensive research has focused on the effect of dietary intake of a variety of substances on serum lipid

concentrations. Diet has also been implicated in gallstone formation. Gradual reduction in total calories to attain ideal weight, increased intakes of complex carbohydrates and indigestible fiber, as well as low intakes of simple sugars, appear to lower the risk of gallstone disease by decreasing serum lipid concentrations (20-23). In the past much attention has been focused on dietary cholesterol and saturated fatty acid (SFA) intakes and their effect on serum cholesterol and triglyceride concentrations (24, 25). Examination of dietary protein source, animal or vegetable, has shown that protein may alter serum lipid concentrations. Studies using man or experimental animals have confirmed that dietary vegetable proteins are effective in lowering serum lipid levels when compared to animal proteins (26-31). Protein source has also been shown to effect gallstone formation in laboratory animals such as hamsters (20, 32).

The purpose of this study was to determine the influence of dietary protein sources on serum cholesterol levels and the major bile constituents. The specific objectives of this study were to:

1. Determine the effect of dietary vegetable (cottonseed) and animal (casein) proteins on cholesterol gallstone formation in hamsters.
2. Determine the effect of dietary vegetable (cottonseed) and animal (casein) proteins

on the three major bile constituents: BAs, cholesterol and PLs.

3. Examine the effect of dietary vegetable and animal protein on the total cholesterol concentration in both serum and the HDL fraction.

4. Determine if a correlation exists between contents of bile components and serum lipid concentrations with the different diets.

5. Determine if the manufacturer's processing of the diets (pelleted vs. powdered) has any effect on growth and gallstone formation.

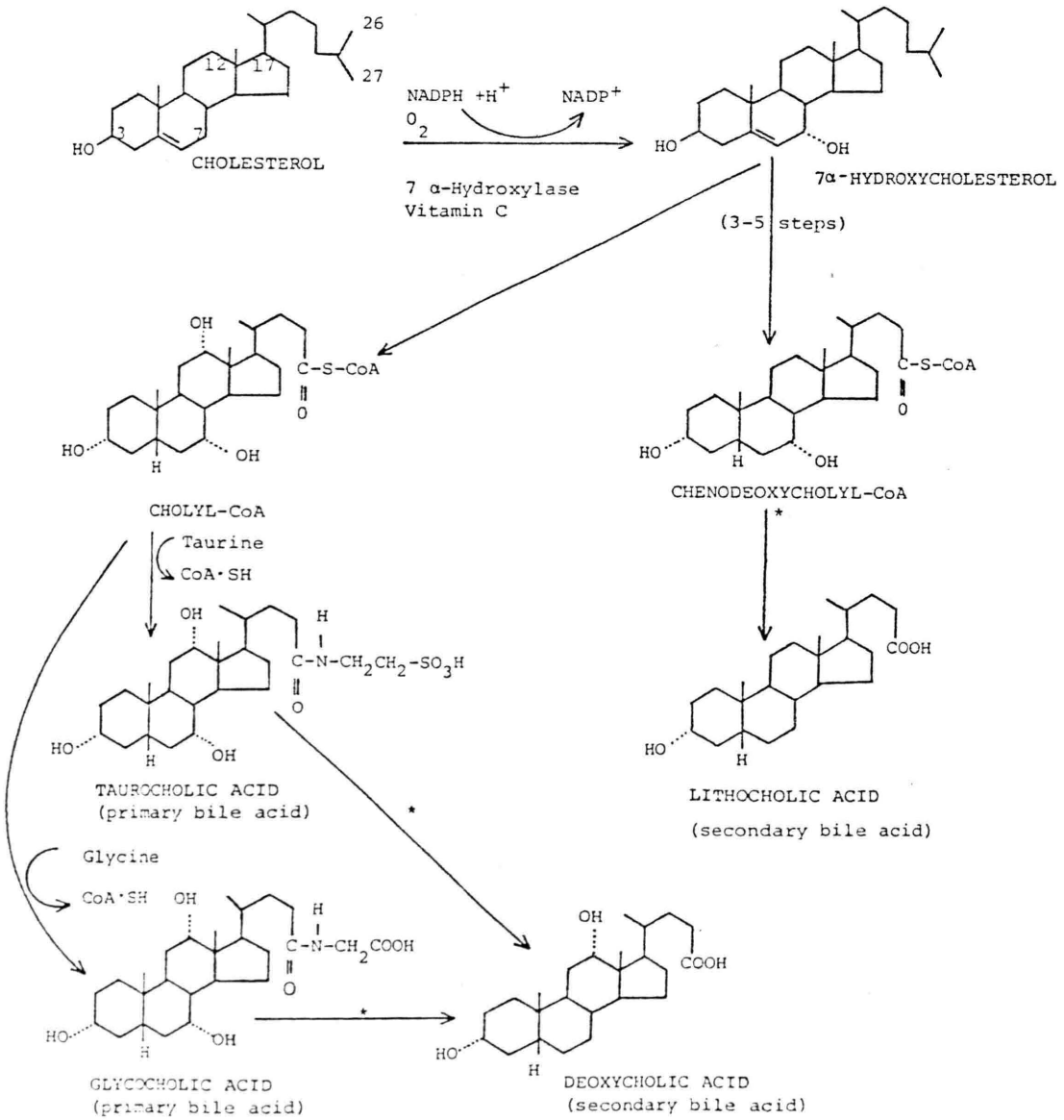
CHAPTER 2

REVIEW OF LITERATURE

Bile Synthesis, Metabolism and Functions

Cholesterol, synthesized primarily in the intestine and the liver, is the precursor for important metabolic products which are formed from the steroid nucleus. In terms of quantity, BAs are the main product of cholesterol degradation. Bile acids are classified as primary or secondary BAs.

The primary BAs are cholic and chenodeoxycholic acid. These acids are synthesized in the liver in three major steps. In the first reaction hydroxyl groups are added to the steroid nucleus at the C-7 and C-12 carbon positions in the alpha orientation. A beta-hydroxyl group is added at the C-3 position and is converted to the alpha position. The reduction forms dihydroxy chenodeoxycholic and trihydroxy-cholic acid. The enzyme 7-alpha-hydroxylase (EC 1.14.13.17), released from the endoplasmic reticulum of the hepatocyte catalyzes this reaction. Nicotinamide adenine dinucleotide phosphate-H (NADPH), oxygen, and ascorbic acid are required. Formation of the 7-alpha-hydroxycholesterol is the rate limiting step in BA synthesis (4) (see Figure 1).



*Deconjugation and 7 α-dehydroxylation by microbial enzymes

Fig. 1. Biosynthesis and Degradation of Bile Acids

In the second step the double bond of the steroid nucleus at the C-5 and C-6 position is reduced.

In the final step the hydrocarbon chain of cholesterol is shortened to five carbons by oxidative and thiolytic cleavage. A carboxyl group (CoASH) is added at the C-26 position. The CoASH is then replaced by either of the two amino acids, glycine or taurine, in peptide linkage at the C-24 position. This reaction requires ATP, CoA, and Magnesium (Mg^{++}) as cofactors.

After conjugation with glycine or taurine, the BAs are secreted from the liver cell into hepatic bile ducts in a mixture of glyco- and taurocholic acids and glycochenodeoxycholic and taurochenodeoxycholic acids. The ratio of glycine to taurine conjugates is 3:1. Conjugated BAs can pass from the liver directly into the duodenum through the common bile duct, or may be stored in the gallbladder for future use in the digestion of dietary fats.

Secondary BAs are deoxycholic and lithocholic acid. Primary BAs are converted by bacterial action to secondary BAs while transported through the intestine. The reaction to form secondary BAs involves hydrolysis of the amide peptide linkage and release of glycine and taurine (see Figure 7) as well as the removal of the hydroxyl group at the C-7 position. The newly formed secondary BAs are absorbed in

the intestine and carried back to the liver through the portal blood stream (33).

Bile secretion by the liver is stimulated by the hormone hepatocrinin. The bile released by the liver and collected in the gallbladder contains both primary and secondary BAs. Gallbladder bile in addition contains cholesterol, PLs, bilirubin, water, and salts. Sodium and potassium are present in significant quantities in bile (0.65%). Gallbladder bile is alkaline and has a pH of 6.9-7.7. For this reason it is assumed that BAs are actually present in the form of salts and are, therefore, frequently referred to as bile salts (34, 35).

As bile is collected and stored in the gallbladder, water is absorbed through the cell walls and the bile becomes more concentrated. Gallbladder bile is transferred into the intestine via the cystic duct, after stimulation by cholecystokinin which is secreted from the small intestine in response to dietary amino acids and fats.

In the intestine BAs combine with dietary lipids (cholesterol, PLs, and triglycerides) to form mixed micelles, an important step in lipid digestion (35, 36). The mixed micelles release their contents for absorption at the brush border where BAs are deconjugated from glycine and taurine. The deconjugated BAs are combined with albumin

and carried back to the liver in the portal blood. In the liver the recycled BAs are separated from albumin and re-conjugated with glycine and taurine and resecreted into bile. This recycling process is known as the enterohepatic circulation (EHC) which provides an important portion of the total BA pool (4). The BA pool of approximately 3-5 gms must be kept at an optimal level. Only a small amount of BAs (approximately 1%) are lost in the feces in the form of lithocholic acid. This loss is replaced through de novo BA synthesis from cholesterol. Cholesterol degradation to BA is the major pathway of cholesterol excretion, and is therefore very important for the maintenance of normal cholesterol levels in serum (4, 34, 35).

Synthesis of cholesterol by the liver is controlled by dietary intake of cholesterol and by BAs in the EHC. Changes in the rate of synthesis of BAs are generally parallel to changes of cholesterol synthesis in the liver. The rate limiting step of BA biosynthesis is at the 7-alpha-hydroxylase reaction; in the biosynthesis of cholesterol it is at the 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase step.

The activities of these two enzymes change in parallel, and it is not clear if synthesis of BA is inhibited primarily at the 7-alpha-hydroxylation step, or at the

HMG-CoA reductase step. Bile acid synthesis is regulated by the amount of BAs returned to the liver from the intestine. Bile acid synthesis decreases as increased levels of BAs return to the liver from the intestine. As cholesterol absorption increases, BA synthesis increases (19, 33).

Bile secretion from the liver is stimulated by the hormones hepatocrinin and cholecystokinin which are secreted in response to foods in the intestine. Hepatocrinin activates release of bile into the gallbladder. Cholecystokinin stimulates the gallbladder to start rhythmic contractions which expel bile through the Sphincter of Oddi into the intestine (4, 34, 35). Other factors which control BA synthesis are dietary components such as cholesterol, saturated fats, proteins, carbohydrates, fiber, and vitamins. Diseases characterized by faulty lipid metabolism and hormone changes have been observed to alter BA formation. Bile acid synthesis also responds to drug therapies which alter cholesterol synthesis as well as cholesterol and BA elimination in feces (4, 7, 14, 33).

Bile acids have three important functions in the body: emulsification of fats, neutralization of gastric acid, and excretion of a variety of materials from the body. The primary function of BAs is to emulsify partially digested fats in the small intestine. Bile acids lower the surface

tension at the oil-water interface, and solubilize fatty acids and water-insoluble soaps by forming mixed micelles with fatty acids, cholesterol and PLs. This allows for the absorption of fatty acids, cholesterol, fat soluble vitamins, and monoglycerides at the brush border. Bile acids make the lipid molecule more accessible to action by pancreatic lipase for cleavage of fatty acids to form di- and monoglycerides. The digestion of other food constituents is also aided by the action of BAs as they prevent the coating of food particles with fat which makes them less available for breakdown and allows increased bacterial action, causing putrefaction (35, 37).

The second important function of BAs lies in the neutralization of gastric acid in the intestine. Bile acids represent a major reservoir of alkali (35).

The third function of BAs is to aid in the removal of drugs, toxins, bilirubin, and various inorganic substances from the body. The most important excretory function of BAs is to serve as a catabolic product of cholesterol removal from circulation in the body. The effect of BAs on cholesterol metabolism is to maintain a balance in which the rate of cholesterol synthesis and absorption tends to equal the rate of excretion and degradation under diverse physical conditions. This way BAs can minimize either accumulation of cholesterol in plasma and tissues, or a

deficiency of cholesterol needed for various metabolic functions. Disturbances of this equilibrium between cholesterol and BAs may lead to gallstone disease (38).

Cholesterol is insoluble in the aqueous medium of the bile. It can be kept in solution by forming mixed micelles with bile acids, PLs (primarily lecithin), water, and salts. Mixed micelles, however, have a limited capacity to keep cholesterol in suspension. The "cholesterol-holding capacity" depends on the proportion of cholesterol to BAs and PLs (14). Redinger and Small (39) have devised a triangular coordinate (shown in Figure 2) which illustrates the relative proportions of the three major bile constituents. The normal ratio of cholesterol to PLs and BAs is 5-15-80% respectively. As cholesterol concentration in bile increases and BA and PL concentration decreases the bile becomes saturated or supersaturated and is referred to as lithogenic bile. Presence of lithogenic bile is the first stage of gallstone formation. The second phase for gallstone formation is nucleation, which may be heterogeneous or homogeneous in nature. In heterogeneous nucleation some foreign particles such as bacteria, cell fragments, ova, parasites, or metal fragments form the nucleus around which cholesterol crystals slowly aggregate or add to the surface of the nucleus. Homogeneous

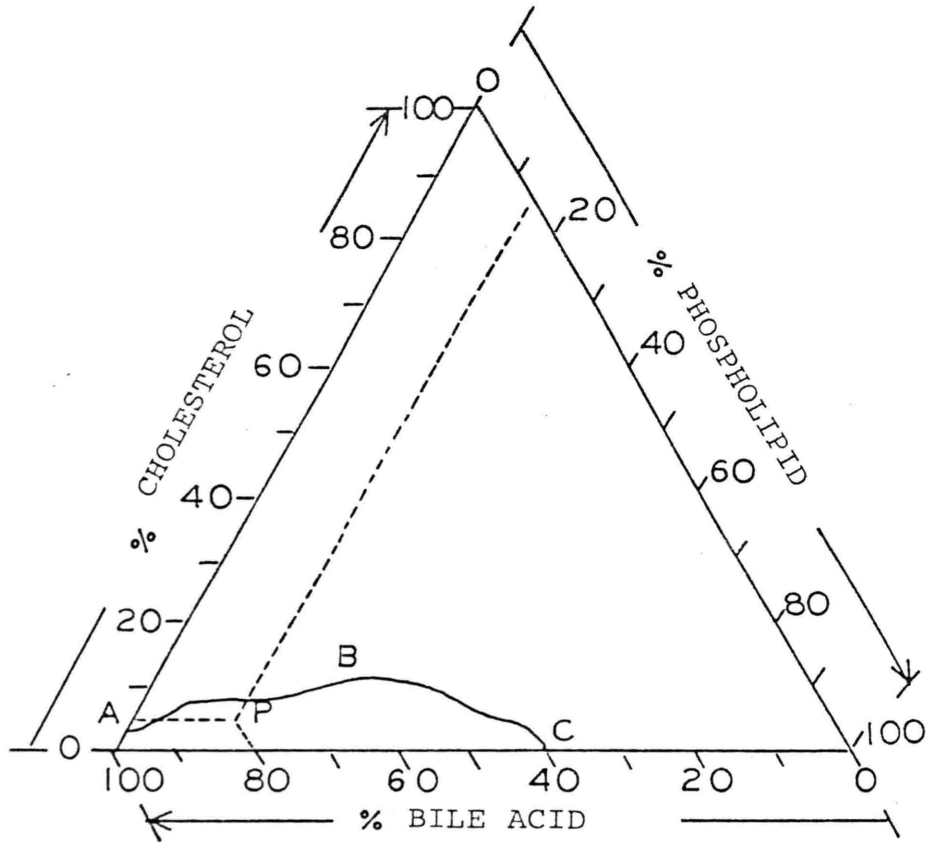


Fig. 2. Triangular Coordinates.

Triangular Coordinates representing three major bile components (bile acid, phopsholipid and cholesterol), expressed as molar percent of total bile sample. Line ABC represents maximum solubility of cholesterol in varying mixtures of bile acids and phospholipids. Point P represents bile composition containing 5% cholesterol, 15% phopholipid and 80% bile acid, which falls in a zone of micellar liquid phase. Bile, having a composition falling above line ABC would contain excess cholesterol in either supersaturated or precipitated form. (Modified from Redinger and Small, 38).

nucleation of cholesterol occurs spontaneously and proceeds at a very rapid rate. The third step in gallstone formation is the growth of the nucleus and microlith to symptomatic size. Small stones in the gallbladder may be expelled or may remain undetected for extended periods of time. Only when bile ducts become obstructed or inflamed, due to the friction of the stones, may clinical symptoms arise (2, 4).

The complete mechanism leading to lithogenic bile and gallstone formation is not clear. The major defect seems to occur in the liver cell and is intimately related to the control mechanism of cholesterol and BA synthesis discussed earlier (2, 34, 36, 37).

Etiology of Gallstone Disease

Cholesterol gallstone disease (GSD) is a multifactorial disease. Several etiological factors appear to promote development of the disease. Age, sex, race, ethnic origin and geographic location influence incidence of GSD.

Gallstone disease is rarely found in humans before 20 years of age. It predominates in premenopausal females, but past the age of 50 years afflicts men and women to the same extent (4, 10). White Anglo-Saxons seem to be at higher risk than persons of black, Spanish or Oriental origin. The Pima Indians of North America report an unusually high incidence of GSD among their middle-aged

women (5). Cholesterol gallstone disease predominates in affluent Western societies who are known to live on high intakes of saturated fats and animal proteins. Oriental and African populations, with vegetables as the main dietary staple, rarely develop cholesterol gallstones. Gallstones found in these countries are mostly pigment stones which are not formed due to faulty cholesterol or BA mechanisms (37).

Several disease states, characterized by defects in lipid metabolism also appear to increase the risk for gallstone disease. An excess amount of plasma lipids, 12-14 hours after eating, indicates diseases known as hyperlipoproteinemias (HLP). Hyperlipoproteinemias are classified into six types according to the predominant lipoprotein present in plasma (40) (i.e., chylomicrons, very low density lipoproteins [VLDL] or low density lipoproteins [LDL]). Serum lipid concentrations of patients with HLP type IIa (elevated serum LDL concentrations), HLP type IIb (high serum concentrations of both LDL and VLDL), and HLP type IV (high VLDL concentrations) have been compared to those of subjects with normal serum lipid concentrations (11-14, 39). These studies showed that men and women with HLP type IV, between 40 and 59 years of age, had nearly twice the incidence of GSD compared to healthy subjects. The composition of BAs in patients with HLP was found to differ from that in normal

subjects. The ratios of cholic to chenodeoxycholic acid and of glycoconjugated to tauroconjugated BAs were found to be higher in patients with HLP compared to normal persons (41). Tauroconjugated BAs are more polar and therefore can attract a nonpolar lipid such as cholesterol and keep it in solution more effectively than the less polar glycoconjugated BAs. Patients with type IV HLP were also observed to have increased cholesterol synthesis, due to a twofold increase in HMG CoA reductase activity, when compared to healthy subjects. In summary, the researchers concluded that an association between HLP and GSD seems to exist.

A high rate of GSD has been observed in obese people (11, 12, 33). Supersaturated bile is frequently found in obese patients and it is believed to be due to increased cholesterol synthesis. Obesity, combined with an oversensitive feedback mechanism, seems to be one factor which plays a role in the development of GSD in Pima Indian women. Even in very young women obesity is common among this tribe, and these young women have been observed to have gallstone free lithogenic bile (5).

Diabetes and glucose intolerance (as seen in type IV HLP) appear to increase risk of GSD. It has been hypothesized that this is due to defective carbohydrate metabolism, which in turn may lead to increased serum lipid

concentrations (4, 33, 42). Impairment of gallbladder contractility has been observed in diabetic patients and could possibly lead to gallstone formation due to prolonged storage of bile in the gallbladder (2). In animal studies, sucrose feeding has been observed to decrease chenodeoxycholic acid and lecithin in bile, therefore altering the relative cholesterol concentration in bile (13, 42).

Increased occurrence of GSD has been associated with pregnancy and contraceptive therapy. Observation in the Framingham Study (10) and the Boston Collaborative Drug Surveillance Program (43) indicated that pregnant women and menopausal women receiving estrogen treatment were at higher risk for GSD incidence. Plasma lipoprotein concentrations (especially VLDL) are frequently elevated in pregnant women. Hormonal changes due to pregnancy may also affect gallbladder contractility. Researchers (4) were able to alter bile flow rates in mice through administration of steroid hormones. Estrogen increased bile flow rates in male mice, while androgen increased bile flow rates in female mice.

Other medical treatment such as ileal resection or bypass surgery may be predisposing factors to GSD by interfering with the reabsorption of BAs into the EHC and consequently lowering the BA pool (25, 44). Gallstone disease is also reportedly higher in patients with liver

disease. Cirrhosis of the liver has been associated with smaller than normal BA pools and above normal levels of BA in serum (4). The range of incidence of GSD in carcinoma of the gallbladder is between 65 and 100% in various reports, however patients with GSD only rarely develop cancer of the gallbladder (4).

Treatment of various diseases may also lead to gallstone formation. Hypolipidemic drugs administered in treatment of atherosclerosis have been observed to increase the cholesterol concentrations in bile relative to BAs and PLs. Clofibrate, nicotinic acid and other drugs increase excretion of cholesterol in feces and BAs (25, 45). As serum cholesterol levels decrease, cholesterol is mobilized rapidly from body tissue pools and secreted into bile faster than compensatory amounts of BAs can be synthesized. This may lead to supersaturated bile and possible gallstone formation. Because of these side effects drugs interfering with cholesterol and BA synthesis are no longer recommended for routine treatment of hyperlipidemic disorders (15, 16, 35, 43, 44).

Dietary Effect on Gallstone Disease

Clinical and epidemiological data have indicated that dietary components have a definite influence on cholesterol and BA metabolism. This in turn may affect gallstone

formation. Dietary and endogenous cholesterol in the liver can follow one or more pathways: synthesis of new lipoproteins, conversion and elimination as BAs and direct excretion into bile. It is believed that dietary and newly synthesized cholesterol in the liver is first incorporated into plasma lipoproteins before it is catabolized and excreted into the bile (46). The rate of cholesterol synthesis in the liver is determined by the amount of dietary cholesterol removed by the liver and by the rate of lipoprotein synthesis (7). In times of stable dietary cholesterol intakes, increased synthesis of plasma lipoproteins may be associated with increased hepatic cholesterol synthesis. If dietary cholesterol intake rises, need for de novo cholesterol synthesis declines. It has been observed that there is a prompt mobilization of cholesterol from tissue stores if plasma cholesterol concentration is reduced. The mobilized cholesterol is carried by plasma lipoproteins to the catabolic pool in the liver (47). Plasma lipoproteins function to transport lipids in a water-soluble form. Chylomicrons carry dietary triglycerides from the intestine to extrahepatic tissues for utilization and storage. Very low density lipoproteins, containing primarily triglycerides, are made in the liver. Low density lipoproteins are derived from catabolism of VLDL.

High density lipoproteins are synthesized in the liver. Most of the cholesterol in plasma is carried by LDL and HDL. Cholesterol is carried from the liver to peripheral tissues by LDL, which regulates de novo cholesterol synthesis in nonhepatic tissues. High density lipoproteins promote cholesterol transport of free cholesterol from peripheral tissues back to the liver (19). The amounts of LDL and HDL in plasma and their relative ratio to each other are reliable predictors of atherosclerosis. High concentrations of LDL have been associated with an increased risk of atherosclerosis, whereas high concentrations of HDL appear to provide protection from the disease (17).

The apparent relationship between dietary cholesterol intake and atherosclerosis has stimulated much research regarding the effects of various amounts of dietary lipids in research animals and man (48-50). To a certain degree man can respond to excessive cholesterol intakes with increased cholesterol excretion and suppressed endogenous cholesterol synthesis. Continuous high cholesterol intakes, however, have been associated with increased cholesterol accumulation in plasma and in various tissues and may lead to formation of lithogenic bile (34). Grundy (24) studied the effects of dietary polyunsaturated fatty acids (PUFA) on lipid metabolism in humans. It was found that feeding of

PUFA increased the excretion of endogenous neutral and acid sterols. Diets containing 40% of the total calories as fat (lard) resulted in increased secretion of cholesterol and a higher incidence of gallstone disease. Other studies (51, 52) confirm that the ratio of PUFA to saturated fatty acids (P/S) is an important factor in the control of plasma lipid concentrations. High intakes of PUFA are recommended to reduce plasma lipid concentrations of VLDL,LDL and total cholesterol in patients with hyperlipoproteinemias. In some patients this treatment has been observed to cause lithogenic bile. It is suggested that a person attain near ideal weight before initiating a change in PUFA intake, and increase PUFA intake slowly until a P/S ratio of 2:1 is reached (25).

Dietary carbohydrates can also influence plasma lipid concentrations. Carbohydrates are partly converted to endogenous triglycerides in the liver and are transported as VLDL in the blood stream. Insulin stimulates lipogenesis and through cAMP inhibits lipolysis (35, 42). Simple sugars, especially glucose and sucrose, are more readily available for conversion to triglycerides than are complex carbohydrates.

Dam (20) and Christensen et al. (53) developed a diet for hamsters which contained 74.3% sucrose. In combination

with casein as a protein source this diet is nearly 100% effective in producing gallstones in hamsters. When sucrose was replaced with a complex starch (rice starch, whole wheat, rolled oats or grain sorghum) gallstone formation was inhibited and previously developed stones could be regressed. Diets high in complex carbohydrates and low in saturated fat and cholesterol have also resulted in a significant reduction of VLDL and LDL in hyperlipidemic and normolipidemic subjects (54, 55). Addition of indigestible fiber to the diet has been shown to lower plasma triglyceride concentrations and to decrease the risk for atherosclerosis. Fiber can combine with cholesterol and BAs in the intestine to form complexes which are excreted in feces. Vegetables in the diet seem to lower total plasma cholesterol concentration, and different types of vegetables were associated with a lowering of specific plasma lipoproteins. People adhering to strict vegetarian diets reportedly have lower plasma lipoprotein concentrations than people eating mixed diets (56).

The source of dietary protein, vegetable or animal, appears to be a major influencing factor on plasma lipid concentrations. Extensive research in recent years has focused attention on dietary protein sources.

Observation that the "Dam diet" was most lithogenic when casein was used, led to further examination of the

dietary protein source. Kritchevsky and Klurfeld (32) observed that vegetable protein diets (soybean), when fed to hamsters, reduced gallstone formation and helped regress gallstones which had already formed. In several studies with rabbits, Carroll (29, 57) concluded that protein from vegetable sources (soybean) tended to protect animals from atherosclerosis. Forsythe (58) fed pigs with diets containing either soybean meal or casein as well as various concentrations of fats. Results showed that total plasma and HDL cholesterol concentrations were lower in pigs fed plant protein, compared to those eating animal protein. Roy et al. (59) studied mice fed cholesterolemic diets containing various protein sources and trypsin inhibitor. The animals fed soy diets had lower plasma cholesterol and higher intestinal BA concentrations than mice fed casein diets. Sirtori et al. (31) compared the effect of soy protein to animal protein on 42 hypercholesterolemic subjects fed diets containing various amounts of fat. Results showed that soy protein diets were hypocholesterolemic at different P/S ratios, but became less effective in diets with low P/S (0.1) ratios. The researchers concluded that soybean diets have therapeutic value in reducing plasma lipid levels in patients with hyperlipoproteinemia (type II a and b). A possible mechanism for this hypocholesterolemic effect of

plant protein may be the increased fecal excretion of neutral sterols. Van Raaij (60) fed young, healthy male and female students identical diets containing either casein or soy protein. After six weeks of feeding he found that total serum cholesterol did not significantly change in either group. There was however a significant reduction in LDL cholesterol and an increase in HDL cholesterol in the group fed soy protein.

Kritchevsky (26) obtained different results when he fed various combinations of dietary amino acids to rabbits. He hypothesized that the ratio of lysine to arginine may be an important factor in the mechanism determining atherogenicity of protein. Lysine was found to inhibit liver arginase activity. When dietary lysine was higher in concentration than arginine, the arginine was incorporated into arginine-rich lipoprotein E, which has been observed to contribute to atherosclerosis. The lysine:arginine ratio of casein is 2.0 and that of soy protein is 0.9. Kritchevsky found that the addition of enough lysine to soy protein diets to raise the ratio to the same level as in casein made these diets more atherogenic. Huff and Carroll (61) carried out more detailed experiments on rabbits to further investigate the relationship between dietary amino acid composition and plasma cholesterol levels. Although these researchers failed

to confirm Kritchevsky's findings concerning lysine:arginine ratio, dramatic changes were observed with various manipulations in the amounts of essential and nonessential amino acids in the diet. The results of this study led to the conclusion that an interaction of essential and nonessential amino acids is an important influencing factor on plasma cholesterol concentrations. Park and Liepa (62), studying rats fed various vegetable and animal proteins, confirmed that amino acid combinations do affect plasma lipid concentrations. Higher serum VLDL and LDL concentrations in rats fed animal protein compared to those fed plant protein have also been documented (30). A high correlation between dietary arginine and serum triglyceride levels was established in this study. Although the mechanism is poorly understood there is evidence that dietary protein source is an influencing factor in atherosclerosis and in gallstone disease.

Few studies have examined the importance of vitamins and trace minerals on plasma lipid concentrations and on gallstone formation. Several vitamins are important cofactors in BA metabolism. Vitamin C is a cofactor in the 7-alpha-hydroxylase step of BA synthesis. Chronic vitamin C deficiency was observed to decrease BA synthesis in guinea pigs (63). Niacin is needed for synthesis of NADPH,

which is also a cofactor in BA synthesis. Vitamin B_6 is necessary for the conversion of methionine to cystathionine. Malfunctions in this pathway have been associated with atherosclerosis (64). Feeding of vitamin K_1 and K_2 in rats seems to increase BA excretion in feces. Vitamin A deficiency in rats decreases cholesterol synthesis at the squalene step (33). In summary, it may be concluded that diet as a whole is an important factor in lipid metabolism and gallstone disease. Dietary protein, however, has been shown to play a key role. Interactions between protein and other dietary components such as fat, carbohydrates, and fiber are involved in this mechanism. Variations in the catabolism, absorption and synthesis of these dietary components in individuals also imply that multiple etiologies exist in the development of gallstones.

Treatment and Prevention of Gallbladder Disease

At this time the most common treatment for GSD is surgical removal of the gallbladder. The gallbladder is not an essential organ since enough BAs can be contained in the enterohepatic circulation for proper function of the BA mechanism.

Research involving regression of formed gallstones is still in the experimental stages. Dissolution of gallstones

has been accomplished in some animal and human studies with dietary treatment and with pharmacological agents (20, 32).

Normal bile contains enough BAs and PLs to keep cholesterol in suspension. It has been hypothesized that lithogenic bile may become unsaturated if the bile salt pool could be expanded through feeding of bile salts. When chenodeoxycholic acid and cholic acid were administered to women suffering from cholelithiasis, previously saturated bile became unsaturated with chenodeoxycholic acid. Cholic acid had no effect on cholesterol concentration in bile (4). Gallstones were regressed in 6-18 months of chenodeoxycholic acid therapy (65, 66). Ahlberg et al. (65, 66) observed that hepatic cholesterol concentration was approximately 20% higher in patients with GSD, compared to healthy subjects. Treatment of these GSD patients with chenodeoxycholic acid resulted in normalization of hepatic cholesterol concentration. Bile acid treatment reduced HMG CoA reductase activity by 40% in some of the subjects treated. Cholic acid had no effect on hepatic bile saturation. In another study (67) it was found that feeding of chenodeoxycholic acid to gallstone patients brought concentrations of phosphatidylcholines, which represent more than 95% of biliary PLs, within normal range.

For treatment of hyperlipoproteinemias, weight reduction to near ideal body weight has been found to be most

successful (68). Weight loss is generally accompanied by reduction of serum lipoprotein concentrations to near normal levels, and may therefore also be beneficial in prevention of GSD (69). Weight loss however should proceed slowly to prevent massive mobilization of tissue lipid stores which might lead to increased plasma lipid concentrations and increased risk for GSD (70).

Prevention of GSD may be possible by reducing the risk factors. Maintenance of ideal body weight through appropriate caloric intake is an important first step. Intake of a prudent diet low in saturated fats, animal protein and simple carbohydrates, but rich in all types of complex carbohydrates, vegetable protein and unsaturated fats, is recommended, beginning at an early age. This diet, combined with increased physical activity should greatly aid in maintaining normal lipid metabolism and consequently reduce the risk for atherosclerosis and gallstone disease (71, 28).

Effect of Manufacturing Process on Dietary Protein

Diets in pellet form are more convenient to administer to hamsters. Pelleted diets are less wasteful and are more compatible with the natural instincts (gathering and storing) of the hamsters. Pelleted diets are therefore preferred to powdered diets for hamsters in the laboratory

setting. The pelleting process of a diet subjects food components to an increase in temperature under high pressure. The possibility of a denaturing affect on the proteins exists. When subjected to heat, proteins undergo structural changes, which uncoil protein macromolecules and expose reactive groups. The denatured proteins can interact with each other through a variety of bonds to form aggregates which contribute to opacity, gel structure and water solubility. Denatured protein becomes insoluble in solutions in which the original protein was soluble (72). Solubility of protein may be an important factor in the digestion and absorption of proteins. Decreased availability of protein or lack of specific amino acids may affect lipoprotein synthesis in the liver. The resultant changes of serum lipoprotein concentrations may also lead to GSD.

CHAPTER 3

EXPERIMENTAL DESIGN

Selection of Animal Model

In the affluent societies of North America and Western Europe cholesterol gallstone disease (cholelithias) has been found to occur frequently (2). Cholesterol gallstones can also be induced in some laboratory animals by dietary means. The hamster meets the criteria for an ideal animal model, since its bile and gallstone composition resemble the composition of bile and gallstones found in humans. Hamsters produce sufficient quantities of bile and stones for analysis when treated with specific diets (20, 53). Some differences do exist between gallstone formation in hamsters and humans. In man, gallstone formation increases with advancing age and is found more frequently in female than male patients, particularly during middle age. Hamsters however, form gallstones more rapidly if experimental treatments are initiated soon after weaning, and the incidence of gallstones is higher in male than female animals (2).

Description and Care of Animals

Thirty male hamsters (Mesocricetus auratus, Engle Laboratories, Inc., Farmersburg, Indiana), approximately four weeks old, weighing 65 ± 5 g were utilized for this study. The animals were housed individually in wire cages (small mesh, 17" x 9.5" x 7") in a well ventilated room. Artificial lighting was provided from 7 a.m. to 7 p.m. The body weight of each animal was recorded upon arrival, on the first day of experimental diet, and every seven days thereafter until the time of sacrifice. After the initial weighing the hamsters were randomly placed into individual cages. Fourteen days were allocated as an equilibration time, during which all animals were fed Purina Rodent Chow (diet #5012). Following equilibration the hamsters were fed one of three diets (10 animals per group) for 35 days. Food and water were provided ad libitum throughout the study.

The control diet (Group I) contained casein as a protein source and was fed in a pelleted form. The experimental diets contained either casein or cottonseed as a protein source (Table 1). The experimental casein diet (Group II) was fed in a powdered form, whereas the experimental cottonseed protein diet (Group III) was fed in a pelleted form. The three diets used in this study contained proteins of high purity (isolates which are considered

TABLE 1

COMPOSITION OF DIETS[1]

Ingredient	Group I Pelleted Casein	Group II Powdered Casein	Group III Pelleted CSP[2,3]
	%	%	%
CSP[3]	0	0	20.0
Casein[4]	20.0	20.0	0
Sucrose	74.3	74.3	74.3
Vitamin Mix[5]	0.5	0.5	0.5
Mineral Mix[5]	5.0	5.0	5.0
Choline Chloride	0.2	0.2	0.2

[1]Supplied by U.S. Biochemical, Cleveland, Ohio.

[2]CSP: Cottonseed protein.

[3]CSP isolate was supplied by Oilseed Protein Research at Texas A & M University, College Station, Texas (Table 3)

[4]Casein isolate was supplied by U.S. Biochemical, Cleveland, Ohio (Table 2).

[5]Supplied by U.S. Biochemical, Cleveland, Ohio (Tables 4 & 5).

to be free of carbohydrates, fat and indigestible fiber), at a concentration of 20% of total ingredients (Tables 2 and 3). Vitamins and minerals were added in equal amounts to all three diets (Tables 4 and 5).

Collection and Preparation of Samples for Analysis

Experimental diets were fed for 35 days. The animals were then fasted for approximately 12 hours, water was allowed ad libitum. Sacrifice was performed between 8 a.m. and 11 a.m. The hamsters were anesthetized with ether, after which the abdominal and chest cavities were exposed by making a midline incision through the abdominal wall. Blood was withdrawn by internal cardiac puncture of the right atrium into nonheparinized syringes. Samples were collected in sterile 3 ml polypropylene test tubes, capped, and chilled with ice until completion of the sacrifice. Bile was aspirated from the gallbladder using 50 µl Hamilton syringes, collected in self-closing 1 ml polypropylene centrifuge tubes and chilled with ice until the sacrifice was completed. The gallbladders were visually examined for the presence of stones. Gallstones, if present, are clearly visible to the unaided eye. A dissecting microscope was used for verification of the presence of stones if any question existed. Gallbladders that contained stones were

TABLE 2

COMPOSITION OF PROTEIN IN CASEIN DIET[1]

Ingredient	% of Total
Protein (Nx6.25, as is)	85.4
Moisture	9.8
Ash	1.8
Ether Extractables	1.1
Carbohydrates	1.8
Fiber	0.1
Heavy Metals	10 ppm

[1]Supplied by U.S. Biochemical, Cleveland, Ohio.

TABLE 3

COMPOSITION OF PROTEIN IN COTTONSEED PROTEIN DIET[1]

Ingredient	% of Total
Protein isolate (Nx6.25, as is)	84.8
Moisture	3.7
Ash	6.5
Fat	0.6
Carbohydrate	3.8
Fiber	0.6

[1]Supplied by Oilseed Protein Research Center at Texas A&M University, College Station, Texas.

TABLE 4

COMPOSITION OF VITAMIN MIXTURE

Ingredient	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 (5000,000 U/g)	0.0040
Vitamin E acetate (250 U/g)	0.4850

TABLE 5

COMPOSITION OF MINERAL MIXTURE

Ingredient	Per Cent
$Al_2 (So_4)_3 \cdot 24 H_2o$	0.009
$CaHPO_4 \cdot 2 H_20$	11.280
CaCO3	6.860
$Ca (C_6H_5O_7)_2 \cdot 4 H_20$	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}

excised away from the liver and were stored in saline solution for future reference. After completion of sacrifice, blood samples were centrifuged at 4000 rpm for 30 minutes at 4°C. Serum was collected in closable polystyrene test tubes and frozen at -20°C for later analysis. Bile was also frozen at -20°C for future analysis.

Analytical Techniques

Total serum cholesterol was determined quantitatively using the enzymatic method of Roeschlau (73, Appendix B). High density lipoprotein cholesterol was analyzed using the heparin-manganese precipitation procedure developed by Warnick et al. (74, Appendix C). The three major bile constituents: Cholesterol, BAs and PLs were analyzed quantitatively by the methods of Reyes et al. (75, Appendix D), Turley et al. (76, Appendix E) and Trudinger (77, Appendix F), respectively.

Statistical Analysis

Data obtained using the five different analytical tests were computed and analyzed separately using one-way analysis of variance or the student's t-test(78). Between group differences were tested by using the Newman-Keuls multiple range procedure (78). The relationship between serum cholesterol and bile cholesterol was measured and reported

as a correlation coefficient. A chi-square analysis was performed to investigate the effects of dietary protein on gallstone formation. For all statistical analyses a probability of 0.05 or less was accepted as statistically significant.

CHAPTER 4

RESULTS

The primary objective of this study was to determine the effect of dietary vegetable and animal proteins on cholesterol gallstone formation in hamsters. The specific objectives of this study were to examine the effect of vegetable and animal proteins on the concentrations of the three major bile constituents, and on the concentrations of total cholesterol and high density lipoprotein cholesterol in serum. This study was also designed to determine if a correlation existed between the concentrations of various biliary components and serum cholesterol concentrations in animals fed different diets, and finally to examine if the processing of the diets (pelleted versus powdered) had any effect on hamster growth and gallstone formation.

Effects of Dietary Protein on Cholesterol Gallstone Formation in Hamsters

The results of this study showed a significantly greater ($p < 0.01$) amount of gallstone formation in hamsters fed casein when compared to animals fed cottonseed protein (Table 6). Of eight animals fed pelleted casein (Group I),

TABLE 6

EFFECT OF DIETARY PROTEIN ON BILE CONSTITUENTS IN HAMSTERS FED VARIOUS PROTEIN DIETS

Group	Gall-stones	%	Absolute Concentration (μmol/ml)			Relative Concentration (molar %)		
			Cholesterol	Bile Acid	Phospholipid	Cholesterol	Bile Acid	Phospholipid
I Pelleted Casein (N8)	5	63	3.80 ± 0.48[a]	125.79 ± 15.76*	26.39 ± 3.91	2.40 ± 0.33[a]	82.11 ± 0.56[a]	15.49 ± 0.76[a]
II Powdered Casein (N10)	9	90	2.87 ± 0.91[a]	93.86 ± 13.74	19.57 ± 2.55	2.62 ± 0.37[a]	78.96 ± 3.35[a]	18.42 ± 3.61[a]
III Pelleted Cottonseed (N9)	0	0	1.70 ± 0.57[b]	137.32 ± 9.81	15.38 ± 1.49	0.98 ± 0.17[b]	89.04 ± 0.79[b]	9.98 ± 0.77[b]

*Mean ± Se. Means in the same columns bearing different superscript letters are significantly different ($p < 0.05$)

five had gallstones present in the gallbladders. Nine of the animals fed powdered casein (Group II) were found to have gallstones. The difference between these two groups was not statistically significant. There were no gallstones present in the animals fed cottonseed protein (Group III) (see Figure 3). The Chi square value associated with the global results is: (Chi 2 [df,2] = 15.89; $p < 0.01$).

Effect of Dietary Protein on Biliary Constituents

The concentrations of the three major bile constituents: cholesterol, bile acids and phospholipids were analyzed quantitatively to determine if the dietary protein source had any effect on the bile components. Bile constituents were reported first as absolute concentrations measured in micromoles/ml. The absolute values were then calculated as relative proportions (molar %) (Table 6).

Effects on Biliary Cholesterol Concentration

Absolute biliary cholesterol concentrations ranged from 1.0 µmol/dl in the cottonseed protein to 6.6 µmol/ml in the pelleted casein group. The mean values for the three groups were: 3.80 µmol/ml (SE $\pm$ 0.48) group I, 2.87 µmol/ml (SE $\pm$ 0.91) for group II, and 1.70 µmol/ml

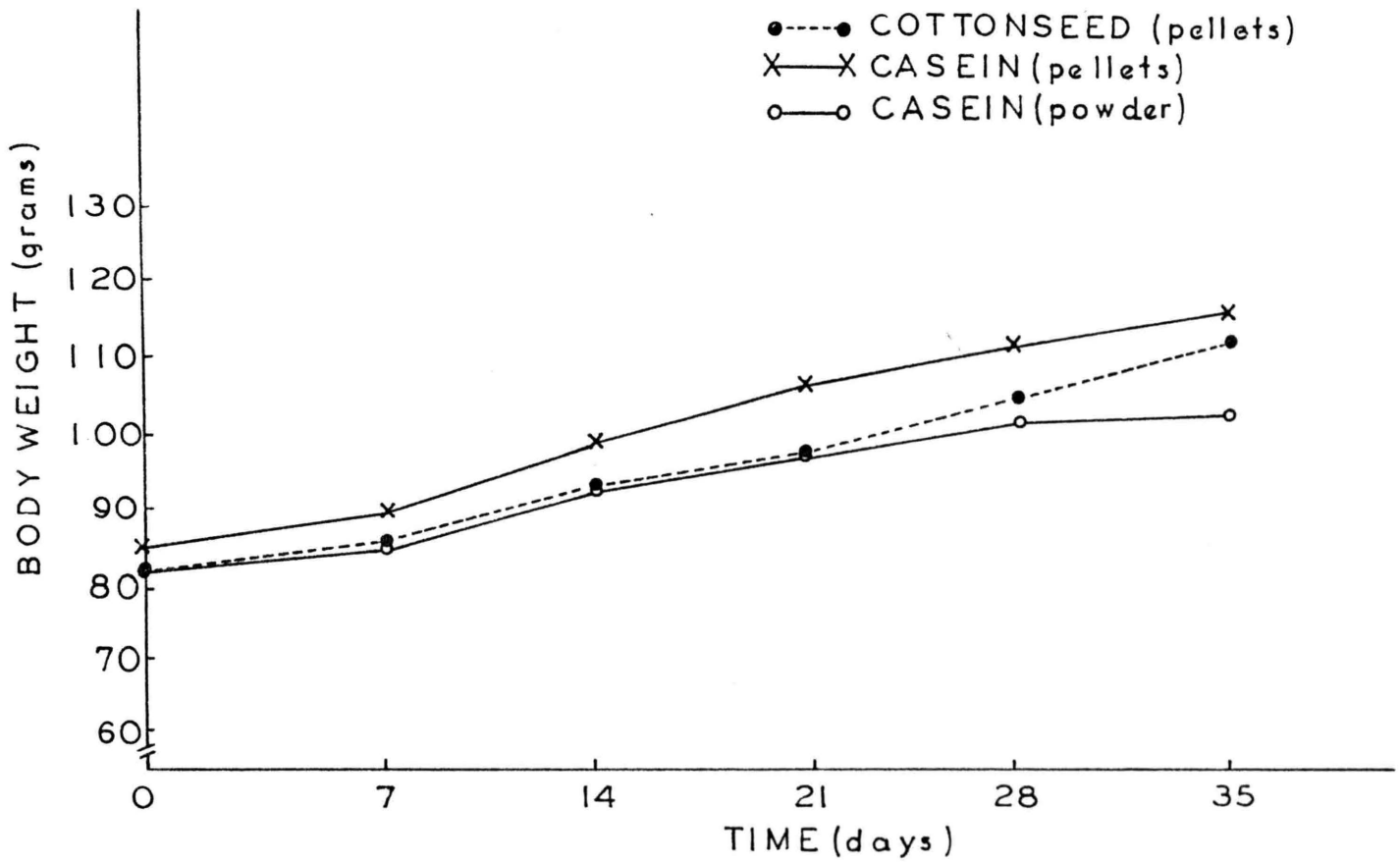


Fig. 3. Weight Changes of Hamsters fed Diets Containing Various Proteins.

(SE ± 0.56) for group III. There was a significant difference between the casein and the cottonseed protein groups ($F(2,18) = 3.96$) $p < 0.05$). The relative concentrations of bile cholesterol were 2.40% and 2.62% for the casein groups and 0.98% for the cottonseed group. The difference between the casein groups vs. the cottonseed group is significant at the $p < 0.01$ level ($F(2,15) = 9.62$).

Effects on Bile Acid Concentration

The means of the absolute concentrations of total bile acids in the bile of the animals of the three different dietary groups were not statistically different from each other. The mean values for group I were 125.79 µmol/ml (SE ± 15.76), for group II 93.86 µmol/ml (SE ± 1.74) and for group III 137.32 µmol/ml (SE ± 9.81). However, when bile acids were expressed as relative values the means of the two casein groups were significantly different from the cottonseed group. The mean value for group I was 82.11%, for group II was 78.96%, and for group III 89.04%. ($F(2,15) = 8.78$; $p < 0.01$). Bile acid concentrations were significantly higher in the cottonseed group when compared to the casein groups.

Effects on Phospholipid Concentration

There was no significant difference in the mean absolute concentrations of phospholipids between the three

dietary groups. The mean values were 26.39 µmol/ml (SE ± 3.91) group I, 19.57 µmol/ml (SE ± 2.55) for group II and 15.38 µmol/ml (SE ± 1.49) for group III. ($F(2,15) = 2.76$; n.s.). When the relative concentration of the phospholipids was calculated there was a significant difference between the groups fed different proteins. The animals fed the cottonseed protein diet had significantly lower relative phospholipid concentrations than those fed casein. Mean relative values were: 15.49% group I, 18.42% in group II and 9.98% in group III ($F(2,15) = 5.10$; $p < 0.05$).

Effects of Diets on Total Serum Cholesterol

The means of the serum cholesterol concentrations in the three dietary groups were not statistically different. The means were 104.73 mg/dl (SE ± 16.44) for group I, 118.36 mg/dl (SE ± 19.00) for group II and 114.09 mg/dl (SE ± 12.51) for group III ($F(2,24) = 0.172$; n.s.). Serum cholesterol concentration was higher in group II than in group III but not significantly so (Table 7).

Effect of Dietary Protein on High Density Lipoprotein Cholesterol

A comparison of the three dietary groups showed no significant difference in HDL concentrations between casein and cottonseed fed animals (Table 7). The mean values were

TABLE 7

TOTAL SERUM CHOLESTEROL AND HIGH DENSITY LIPOPROTEIN CHOLESTEROL CONCENTRATIONS IN HAMSTERS FED DIETS CONTAINING VARIOUS PROTEINS

Group	Diet	Total Cholesterol mg/dl	HDL Cholesterol mg/dl
I	Pelleted Casein	104.73 ± 16.44*	79.76 ± 13.41*
II	Powdered Casein	118.36 ± 19.00	96.90 ± 15.68
III	Pelleted Cotton-seed Protein	114.09 ± 12.51	99.57 ± 11.19

*Means ± SE.

79.76 mg/dl (SE ± 13.41) for group I, 96.90 mg/dl (SE ± 15.68) for group II and 99.57 mg/dl (SE ± 11.19) for group III ($F(2,24) = 0.565$; n.s.).

Correlation of Serum Total and HDL Cholesterol Concentration

When serum cholesterol was correlated with absolute bile cholesterol concentration the correlation coefficient was $r = 0.48$ ($df = 19$; $p < 0.05$). The correlation coefficient between serum HDL cholesterol and bile cholesterol was $r = 0.365$.

Effect of Manufacturers Processing of Dietary Protein on Animal Growth

Mean body weight on day 35 of experimental diet was 102.3 g (SE ± 5.4) for group I, 111.8 g (SE ± 2.28) for group II and 114.9 g (SE ± 5.2) for group III ($p < 0.05$). There was no significant difference ($p < 0.05$) in the final weight of the animals fed either casein or cottonseed protein (see Figure 3).

CHAPTER 5

DISCUSSION AND SUMMARY

In this study gallstone formation was found to be significantly higher in hamsters fed diets containing animal protein (casein) when compared to animals fed diets containing vegetable (cottonseed) protein. In the casein groups 63% (pelleted diet) and 90% (powdered diet) of the animals developed gallstones, whereas in the cottonseed protein group all animals remained free of gallstones. These findings are in agreement with similar data reported by Kritchevsky (32) and Dam (20), who reported increased incidence of gallstone formation with the feeding of diets containing casein compared to vegetable protein (soybean). Mahfouz (79) in a study with hamsters found that soy-protein reduced gallstone formation by one half, and cottonseed protein seemed to inhibit gallstone formation completely in hamsters fed lithogenic diets. In the present study all variables were identical for the three dietary treatment groups, with the exception of the protein source. Therefore the assumption may be made that casein was the causative factor in gallstone development. In the formation of gallstones the relative concentrations of the

major biliary constituents: BAs, cholesterol and PLs are the determining factor (39). As relative cholesterol concentration in bile increases and concentration of BAs and PLs decrease, bile becomes lithogenic. A relatively low BA pool was reported by Grundy (5) studying the Pima Indian women with high incidence of gallstone disease. In this study animals with relatively high biliary cholesterol concentrations and relatively low BA concentrations (casein groups), developed gallstones. Animals with relatively high BA concentrations and normal cholesterol concentrations (cottonseed protein group), showed no gallstone formation.

In man, an association between hyperlipoproteinemia and gallstone disease seems to exist (9, 14). For this reason it was of interest in this study to determine if serum and bile cholesterol concentrations may be related. The correlation coefficient between serum cholesterol and bile cholesterol was $r = 0.48$. Serum cholesterol concentrations in animals fed casein were not significantly different from those in cottonseed protein fed animals. It appears in this study that serum cholesterol concentrations were not greatly influenced by dietary variations. Total serum cholesterol concentrations in the individual animals were well within the normal range (80-200 mg/dl) for

hamsters (80). It may be speculated that these animals have some protective mechanism which allows them to keep serum cholesterol concentrations within normal range through increased cholesterol excretion into bile (as seen in casein group) and increased BA synthesis (as seen in cottonseed group). These data are in agreement with the studies by Ahlberg and Einarsson (14, 13) in which serum triglyceride concentrations, but not serum cholesterol concentrations, were associated with gallstone disease in human subjects.

Serum high density lipoprotein cholesterol was determined in this study to examine if this major cholesterol fraction contributed to increased cholesterol levels in bile. A relationship between serum high density lipoprotein cholesterol and biliary cholesterol was not observed. Serum high density lipoprotein cholesterol (HDL) concentrations were not significantly different between the different dietary groups, and were within the normal range. (30-150 mg/dl) (80). In all groups HDL cholesterol was highly correlated with total serum cholesterol ($r = 0.98$), indicating that HDL is a major cholesterol carrying component in serum. Increased levels of HDL are believed to contribute to higher BA synthesis (19). This could not be substantiated in this study since the animals with high HDL

concentrations had relatively low BA levels and had gallstones present. At this writing comparable research examining the specific aspects of HDL cholesterol in gallstone formation does not exist. Current research (underway at this laboratory) examining HDL cholesterol fractions and subfractions in detail may bring greater understanding to the questions about the complex mechanism of HDL and it's relationship to gallstone formation.

The final aspect of this study was to determine if the manufacturing process involved in diet preparation had any effect on hamster growth or gallstone formation. The final weight of the hamsters in the various dietary groups was not significantly different. Acceptance of diet and resultant weight gain was approximately the same in the two casein groups and in the cottonseed group. Gallstone formation between group I (pelleted casein) and group II (powdered casein) was not statistically different. As a result of these observations it may be concluded that the manufacturing process did not affect the protein quality and the lithogenicity of the pelleted casein diet. Administration of pelleted diets to research animals therefore seems appropriate and may be recommended for future studies.

Summary

The results of this study suggest that proteins from an animal source (casein) can promote gallstone formation in hamsters. Proteins from a vegetable source (cottonseed) appear to protect animals from this disease. Significant changes occurred in the relative concentrations of the three major bile constituents when animals were fed different dietary proteins. Total serum cholesterol and HDL cholesterol concentrations apparently were not affected by dietary protein source. On the basis of the previously discussed results the following conclusions may be formulated:

1. Animal protein in the diet is effective in producing gallstones in hamsters.

2. Vegetable protein in the diet seems to protect against gallstone formation in hamsters.

3. Increased biliary cholesterol concentrations and decreased BA concentrations in the hamsters fed casein diets may be suggested as the mechanism for gallstone formation.

4. Additional data is necessary to draw definite conclusions about the relationship between serum cholesterol and biliary cholesterol and the influence of dietary protein on these parameters.

5. Pelleted diets seem to give identical results to powdered diets in terms of both animal growth and lipid metabolism.

Application of this information to the human situation suggests that substitution of all or part of dietary

animal protein with vegetable protein may be beneficial in lowering the risk of cholelithiasis. Since some of the contributing factors in the etiology of gallstone disease are difficult or impossible to alter (genetics, environmental factors), diet modification could represent a convenient and practical alternative in the prevention of gallstone disease in man.

APPENDICES

APPENDIX A

ABBREVIATIONS

ABBREVIATIONS

LDL	Low density lipoproteins
VLDL	Very low density lipoprotein
HDL	High density lipoprotein
CHD	Coronary heart disease
BA	Bile acid
EHC	Enterohepatic circulation
HMG-CoA	3-hydroxy 3-methylglutaryl Coenzyme A
PL	Phospholipid
HLP	Hyperlipoproteinemia
PUFA	Polyunsaturated fatty acids
cAMP	cyclic Adenosine monophosphate

APPENDIX B

METHOD FOR DETERMINATION OF SERUM CHOLESTEROL

METHOD FOR DETERMINATION OF SERUM CHOLESTEROL

Principle

A single aqueous reagent is used in which cholesterol esters are hydrolyzed to free cholesterol by cholesterol esterase (EC 3.1.1.13). The resulting free cholesterol is oxidized by cholesterol oxidase (EC 1.1.3.6). In this reaction hydrogen peroxide is produced, which transforms methanol to formaldehyde. The formaldehyde reacts with ammonium ions to form a chromogen which has a maximum spectrophotometric absorption at 400 - 420 nm. Samples were analyzed using a Perkin-Elmer Model 552 spectrophotometer.

Reagents

A bmc cholesterol Reagent Set, solutions #1 - 4 (Bio-Dynamics/bmc #124087), was used for this assay.

Prepare as much reagent mix as is needed to process all samples and standards. Reagent is stable for one month if stored in amber bottle at 2 - 8°C.

Table for Reagent Mix

Number of Assays	Solution #1 ml	Solution #2 ml	Suspension #3 ml
10	50	2.5	0.2
50	250	12.5	1.0
100	500	25.0	2.0

Analysis of Sample

1. Bring cholesterol reagent mix to room temperature.
2. Each sample requires a sample blank. Prepare each sample in duplicate. Use glass test tubes (3 ml, 12 x 75 mm).
3. Sample Blank (tube A):
   a. 25 µl sample
   b. 2.5 ml cholesterol reagent mix
   c. vortex
4. Sample (tube B):
   a. 10 µl cholesterol oxidase (#4 from bmc kit)
   b. 1.25 ml reagent mixture from sample blank (tube A)
   c. vortex
5. Incubate sample and sample blank for 60 minutes at 37°C.
6. Zero spectrophotometer with distilled water.
7. Read sample blank and sample at 400-420 nm in spectrophotometer (Perkin-Elmer Model #552).

Color is stable for approximately 2 hours at room temperature.

Calculation

Prepare a calibration curve using Preciset Cholesterol Calibration Solution (bmc Preciset Cholesterol Catalog #125512). Prepare standards using cholesterol solutions in concentrations of 50, 100, 150, 200 and 300 mg/dl. Prepare each concentration in triplicate. Follow steps outlined above for cholesterol determination of sample. Plot obtained absorbance on graph paper against the respective cholesterol concentration.

Calculate:

$$\text{mg cholesterol/dl serum} = \text{Optical Density Sample} \times \frac{\text{Concentration of Standard (mg/dl)}}{\text{Optical Density of Standard}}$$

APPENDIX C

METHOD FOR DETERMINATION OF HIGH DENSITY LIPOPROTEIN CHOLESTEROL

METHOD FOR DETERMINATION OF HIGH DENSITY LIPOPROTEIN CHOLESTEROL

Principle

Manganese chloride and heparin are added to the serum and incubated at 4°C for 30 minutes, to precipitate apoB containing lipoproteins (very low density and low density lipoproteins). Lipoproteins are separated by centrifugation (10,000 rpm for 10 minutes) in a Sorvall RC-5B Refrigerated Superspeed Centrifuge. An aliquot of the supernatant containing high density lipoprotein cholesterol can be quantitated following the same enzymatic procedure outlined for total serum cholesterol analysis (Appendix B).

Reagents

1. Manganese chloride ($MnCl_2$) 1M:
   Dissolve 197.91 g of $MnCl_2 \cdot 4H_20$ in 1 liter (l) distilled water in volumetric flask.
2. Heparin (5000 U/ml):
   Dissolve 1 ml of heparin (40,000 U/ml) in 7 ml of saline (0.15 M).

Analysis of Sample

1. Use 3 ml glass centrifuge tubes

2. Add to tubes:
   a. 100 µl serum
   b. 4 µl heparin solution*
   c. 5 µl $MnCl_2$ solution*
3. Vortex
4. Cover samples and incubate in refrigerator for 1 hour.
5. Centrifuge at 10,000 rpm for 10 minutes at 4°C.
6. Pipette 25 µl of the supernatant into test tubes prepared with bmc reagent.
7. Run enzymatic assay as outlined for total cholesterol determination (Appendix B).

Calculation

Total corrected high density cholesterol (HDL) =

HDL cholesterol x 1.09 dilution factor.

*May be mixed together.

APPENDIX D

METHOD FOR DETERMINATION OF BILIARY CHOLESTEROL

METHOD FOR DETERMINATION OF BILIARY CHOLESTEROL

Principle

The bile sample and a known amount of internal standard (stigmasterol) (Supelco, Inc., #4-7158) is heated with alkali to split fatty acid esters from the cholesterol molecule. The resulting free cholesterol is extracted with chloroform. The chloroform containing the free cholesterol is evaporated to dryness under nitrogen gas. The sample is then reconstituted in a known amount of heptane and analyzed quantitatively using a gas liquid chromatograph (Bendix Model #2500).

Preparation of Standard

A. Reference Standards:

1. Weigh 1.00 mg cholesterol standard (Supelco, Inc. #4-4990), mix with 1.00 ml absolute ethanol (chromatographic grade)
2. Weigh 1.00 mg stigmasterol
   mix with 1.00 ml absolute ethanol
3. Combine 0.50 mg cholesterol with 1.00 mg stigmasterol
   dissolve in a final volume of 1.00 ml absolute ethanol.

4. 1.00 mg cholesterol combined with
   1.00 mg stigmasterol dissolved in 1.00 ml ethanol
5. 2.00 mg cholesterol combined with
   1.00 mg stigmasterol dissolved in 1.00 ml ethanol.

B. Internal Standard:

1. 10.00 mg stigmasterol dissolved in 100.00 ml final volume absolute ethanol.
   This yields a concentration of 1 µg stigmasterol/ 10 µl ethanol. Stable in airtight glassbottle with teflon liner for 1 week at 4°C.

C. Reagents:

1. Ethanolic-Potassium Hydroxide (ETOH-KOH)
2. Absolute Ethanol (chromatographic grade) (ETOH)
3. Potassium Hydroxide (KOH) (mW 56.10):
   Slowly add 3 ml of 50% KOH to 47 ml ETOH = 50 ml final volume.
4. 40% Ethanol:
   Add 40 ml ETOH to 60 ml distilled water.
5. Chloroform distilled in glass (chromatographic grade J. T. Baker, Arlington, Texas).
6. Heptane distilled in glass (J. T. Baker, Arlington, Texas).

Sample Preparation

Use only glass 5 ml centrifuge tubes.

1. Add 50 µl stigmasterol internal standard (1:10) to test tube. (Yields 5 µg stigmasterol.)
2. Add 500 µl ETOH-KOH mixture.
3. Add 5 µl bile.
4. Vortex.
5. Heat the tubes in a 55-60°C water bath for 1 hour. Vortex tubes 3 times at approximately 20 minute intervals during incubation.
6. To the cooled tubes add 2 ml chloroform and 1 ml ETOH and vortex.
7. Centrifuge tubes at 1500 rpm for 5 minutes at 4°C in Sorvall RC-5B Refrigerated Superspeed Centrifuge. The mixture will separate into 2 layers or phases. The top layer is water, the bottom layer is chloroform. The separation is frequently delineated by an opaque membrane-like layer.
8. Aspirate the water layer and the opaque layer with Pasteur pipettes as completely as possible, leaving the chloroform layer intact.
9. Add 1 ml of 40% ETOH, centrifuge at 1500 rpm for 5 minutes and aspirate water layer, leaving chloroform.
10. Repeat step #9 two more times, for a total of four washings.

11. Evaporate the chloroform layer to dryness under stream of nitrogen gas. Place the tubes in a warm water bath (40°) to speed evaporation process. Seal dried tubes with corks. May be stored at room temperature until ready to use.
12. At the time of injection into chromatograph add 15 µl of heptane to the dried tubes and vortex.
13. Inject 3 µl sample into chromatograph (in duplicates).

Analysis of Sample

A. Chromatograph Bendix 2500 Conditions:
   1. Glass column - U-shaped 1 meter long by 2 mm interior diameter (Supelco #2-0610).
   2. Packing 3% SP-2250 on 100-120 Supelco Support (Supelco, Inc. #1-1878).
   3. Oven Temperature 250°C.
   4. Inlet or Injector Temperature 260°C.
   5. Flame Ionization Detector Temperature (FID) 350°C.
   6. Transfer Temperature 340°C.
   7. Input Attenuation X 10.
   8. Recorder Attenuation X 2.
   9. Suppression Range X 10.
   10. Helium (Carrier Gas) 48 psi/tank - 3.5 cc/min Flow Rate Module.

11. Hydrogen 49 psi/tank - 30 cc/min Flow Rate Module.
12. Air 48 psi/tank - 300 cc/min Flow Rate Module.

B. Hewlett Packard Integrated Recorder:

1. Zero 0
2. Attenuation 1
3. Chartspeed 1 cm/minute
4. Peakwidth 0.64
5. Threshold 0

C. Calibration Curve:

1. Inject 1 µl cholesterol reference standard (1 mg/ml). Record retention time and peak area (repeat 3 times).
2. Inject 1 µl stigmasterol reference standard (1 mg/ml). Record retention time and peak area (repeat 3 times).
3. Inject 1 µl of combined cholesterol-stigmasterol standard of all three concentrations prepared. Repeat each 3 times and record results.
4. Calculate calibration curve and record mean peak areas of 1 mg cholesterol with 1 mg stigmasterol in 1 ml ethanol.

D. Injection of Sample:

1. Add 15 µl heptane to dried tubes.
2. Vortex.

3. Inject 3 µl of reconstituted sample (in duplicate).
4. Record retention times and peak areas for stigmasterol and cholesterol.

E. Calculation

1. Record mean area of stigmasterol standard and cholesterol standard.
2. Divide area of stigmasterol of sample tube by mean area of stigmasterol of standard tube = Conversion Factor for Internal Standard (IST) in sample.
3. Divide area of cholesterol in sample by conversion factor = Corrected Area of Cholesterol (unknown).
4. Divide corrected area of cholesterol by mean area of cholesterol standard = µg cholesterol/µl bile.
5. µg/ l x 1000 divided by 386.64 (mW cholesterol) = µmol cholesterol / ml bile.

Example:

150775 ($\bar{x}$ area Stigmasterol) ÷ 168936.66 ($\bar{x}$ area Std.) = 0.89249 (Conversion factor) (CF)

136000 (area cholesterol sample) ÷ 0.89249 (CF) = 152380.95 (Corrected area Cholesterol)

152380.95 ÷ 158545 ($\bar{x}$ area cholesterol standard) = 0.9611 µg cholesterol µl bile

0.9611 x 1000 ÷ 386.64 = 2.485 µmol cholesterol/ml bile

APPENDIX E

METHOD FOR DETERMINATION OF BILE ACID

METHOD FOR DETERMINATION OF BILE ACID

Principle

The 3 alpha-hydroxyl group of the steriod in bile acid is oxidized by 3 alpha-hydroxysteroid dehydrogenase (EC 1.14.13.17) (3 α-HSD) to a keto proup with a simultaneous reduction of nicotinamide adenine dinucleotide (NAD) to NADH. Hydrazine hydrate is used as a ketone trapping agent and the amount of NADH produced is measured to quantitate the total amount of bile acid in the sample.

Reagents

EDTA = disodium ethylenediamine tetraacetate

Tris = tris (hydroxymethyl)-aminomethane

1. Tris-HCl Buffer pH 9.5:
   a. Weigh Tris (0.133 M) 16.11 g.
   b. Weigh EDTA (0.666 mM) 223.0 mg.

   Add Tris and EDTA to 1000 ml distilled H_2O. Adjust pH to 9.5 with addition of concentrated HCl. (Stable for 3 months at room temperature.)
2. Tris-HCl Buffer pH 7.2:
   a. Weigh Tris (0.133 M) 3.64 g.
   b. Weigh EDTA (1 mM) 336.2 mg.

Add Tris and EDTA to 1000 ml distilled H_2O). Adjust pH to 7.2 with addition of concentrated HCl. (Stable for 3 months at room temperature.)

3. Hydrazine Hydrate (1M) pH 9.5:

   a. Hydrazine hydrate (100%)

   b. 2N Sulfuric Acid (H_2SO_4)

   c. Distilled Water

   Under a hood (in ice bath) add 50 ml hydrazine hydrate to approximately 200 ml distilled water. Gradually add 15 ml 2N H_2SO_4 and bring up to 1000 ml with distilled water. This yields a 1M solution pH 9.5 which is stable for 3 months (in amber bottle) at room temperature.

4. Nicotinamide adenine dinucleotide (NAD) 7 mM pH 7.0:

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

   b. Sodium Bicarbonate ($NaHCO_3$).

   Dissolve 464.5 mg NAD in distilled H_2O and bring to a final volume of 100 ml. Adjust pH to 7.0 by adding solid $NaHCO_3$. (Stable for 2 weeks when stored at 4°C).

5. Bile Salt Standard:

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

   b. Methanol (Reagent grade).

Weigh 107.50 mg sodium taurocholate and dissolve in 100 ml methanol. Concentration = 0.2 µmol bile acid per 100 µl solution. (Stable for 4 months when stored at 4°C.)

6. 3 alpha-hydroxysteroid dehydrogenase (3 α-HSD):

   a. 3 α-HSD (Worthington, STDHMP code - #LS00 04910).

   b. Tris-HCl Buffer pH 7.2 (cold).

   Prepare enzyme at a concentration of 2 units of enzyme/ml buffer (stable 1 week, when stored at 4°C). Wrap in foil to keep dark.

   Note:

   Prepare standards in volumetric flasks with ground glass stoppers. Do not use magnetic stirrers with enzyme preparations.

Analysis of Bile Sample

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

1. Mix enough Tris-HCl Buffer pH 9.5 with Hydrazine hydrate and NAD to make Reaction mixture (RM) for all samples.

Number of Tubes	Total Volume (ml)	Tris-HCl 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 culture tubes:

   Sample (in duplicate):

   a. 2800 µl (2.8 ml) RM

   b. 1 µl bile

   c. 100 µl methanol (CH_3OH)

   d. 100 µl 3 α-HSD

   Standard (in triplicate):

   a. 2800 µl (2.8 ml) RM

   b. 100 µl sodium taurocholate standard (equal to 0.2 µmol bile acid)

   c. 100 µl 3 α-HSD

   Reagent Blank (in triplicate):

   a. 2800 µl RM (2.8 ml)

   b. 100 µl CH_3OH

   c. 100 µl 3 α-HSD

   Vortex all reagents before adding enzyme. Vortex again after adding enzyme to insure thorough mixing.

3. Incubate all tubes in water bath (30°C) for one hour then read at 340 nm in Perkin-Elmer Model 552 spectrophotometer. Zero the spectrophotometer with distilled water.

   The delta (Δ) optical density (OD) of the sample and the standard is obtained by subtracting the OD of the reagent blank.

Calculations

OD Standard - OD Reagent Blank = OD for standard

(0.2 µmol conc.)

OD Sample - OD Reagent Blank = OD for sample

$$\frac{\text{Concentration of Standard (µmol)}}{\Delta \text{ OD of Standard}} \times \Delta \text{ OD sample} = \text{µmol Bile Acid/µl Bile}$$

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

APPENDIX F

METHOD FOR DETERMINATION OF BILIARY PHOSPHOLIPID

METHOD FOR DETERMINATION OF BILIARY PHOSPHOLIPID

Principle

Organic phosphorus is extracted from the lipid fraction by digestion with perchloric acid and heat. The free phosphorus reacts with ammonium molybdate-malachite green reagent to form a chromogen which can be read at 660 nm in the spectrophotometer (Perkin-Elmer Model 552).

Reagents

To avoid phosphorus contamination of the sample meticulous laboratory procedures are essential for the successful completion of this assay.

Use only glass distilled water (GDW) for all aqueous solutions.

All laboratory glassware (do not use any plastic) must be acid washed in 10% nitric acid solution and rinsed 3 times with GDW.

Use new 1.0 liter amber reagent bottles, rinse 3 times with GDW.

Use new disposable 3 ml glass culture tubes (12 x 75 mm), rinse 3 times with GDW, dry in oven on foil-lined test tube rack.

Seal with corks or foil, store until ready to use.

Prepare the following reagents:

1. 70% Perchloric Acid
2. 4.2% Ammonium Molybdate in 5N HCL:
   a. To make 5N HCl add 415 ml concentrated HCl to 585 ml GDW.
   b. Add 42 g Ammonium Molybdate to 1000 ml (1L) 5N HCl.
3. 0.2% Malachite Green:
   Dissolve 2 g malachite green in 1000 ml (1L) GDW.
4. No. 2 Whatman Filter Paper.
5. 1.5% Tween 20 (Polysorbate or Span).
6. Standards:
   a. Inorganic Standard K_2HPO_4:
      Dissolve 174.8 mg K_2HPO_4 in 100 ml GDW (final volume). Dilute this solution 1:100 with GDW (1 ml:100 ml). This will yield a working standard at a concentration of 1 nmol phosphorus/10 µl solution. Store at 4°C.
   b. Organic Standard Diphenylphosphinic acid (Aldrich Chemical Company, 99% purity, #10,852-9).
      Dissolve 21.81 mg diphenylphosphinic acid in 100 ml ethanol (final volume). Dilute this 1:10 with ETOH to give a working standard at a concentration of 1 nmol phosphorus to 10 µl solution.

Analysis of Sample

Prepare all samples in disposable glass culture tubes (12 x 75 mm), which have been rinsed and dried as stated earlier.

1. Add 1 µl bile to culture tubes (prepare each sample in triplicate).
2. Pipette aliquots of 50,100 and 200 µl of both inorganic and organic standards into culture tubes (each concentration in triplicate). Each standard yields 5,10 and 20 nm of phosphorus (P) respectively.
3. Evaporate contents of tubes to dryness under a stream of nitrogen gas.
4. Add three empty culture tubes for blanks.
5. Add 50 µl 70% perchloric acid to all tubes (work under a hood).
6. Vortex.
7. Place tubes in a sand bath (200°C ± 20°C) under a hood and digest for 90 minutes.
   (Sample blanks contain only perchloric acid at this point.)
8. While samples and standards are incubating prepare the following reagent:
   Mix three parts malachite green (0.2%) with one part ammonium molybdate (4.2%) reagent. Stir for 30 minutes

with magnetic stirrer. Filter malachite green: ammonium molybdate reagent (MG:AM) mixture 2 times through No. 2 Whatman filter paper. (Color should be brown-amber, if green, contamination is present). Prepare only enough reagent to be used immediately, do not store.

9. After 90 minutes incubation remove tubes carefully from sandbath and let cool.
10. Add 400 µl GDW and vortex.
11. Add 2 ml MG:AM reagent followed immediately by 100 µl of 1.5% Tween and vortex. (Work only 2 or 3 tubes at a time.)
12. Read at 660 nm as soon as possible in spectrophotometer (no later than 60 minutes after mixing). Spectrophotometer has to be zeroed with blanks.

Calculations

$$\text{nmol phosphorus/µl bile} = \frac{\text{Concentration Standard (nmol)}}{\text{Optical Density (OD) Standard (nm)}} \times \text{OD Sample}$$

The average concentration of the combined three standards 5,10 and 20 nmol = 11.67 nmol phosphorus for both the organic and inorganic standards.

11.67 divided by mean OD of the three standards = Constant Standard (inorganic or organic).

Mean OD sample times constant inorganic standard = nmol P per 0.5 ml x 2 = nmol/ml.

$$\text{nmol P/ml bile} = \text{nmol P/}\mu\text{l bile} \times \frac{1000}{1000}$$

Example:

35 ÷ 3 = 11.67 ÷ .410 (mean OD inorganic standard) = 28.45 = Constant inorganic standard.

Mean OD sample 0.23 ÷ 28.45 x 2 = 12.94 nmol P/ml bile.

REFERENCES

REFERENCES

1. Admirand, W. H., and Small, D. M.: Physiochemical basis of cholesterol gallstone formation in man. J. Clin. Invest. 47:1043, 1968.

2. Bennion, L. J., and Grundy, S. M.: Risk factors for the development of cholelithiasis in man. N. Eng. J. Med. 21:1161, 1978.

3. Ahlberg, J., Angelin, B., and Einarsson, K.: Hepatic 3-hydroxy-3-methylglutaryl coenzyme A reductase activity and biliary lipid composition in man: relation to cholesterol gallstone disease and effects of cholic acid and chenodeoxycholic acid treatment. J. Lip. Res. 22:410, 1981.

4. Brobeck, J. R.: Best and Taylor's Physiological Basis of Medical Practice. Williams and Wilkin's Company, Baltimore, Maryland, 1979.

5. Grundy, S. M., Metzger, A. L., and Adler, R. D.: Mechanism of lithogenic bile formation in American Indian women with cholesterol gallstones. J. Clin. Invest. 51:3026, 1972.

6. Hill, G. L., Mair, W. S., and Goligher, J. C.: Gallstones after ileostomy and ileal resection. Gut 16: 932, 1975.

7. Dietschy, J. M.: The role of bilesalts in controlling the rate of intestinal cholesterogenesis. J. Clin. Invest. 47:286, 1968.

8. Ramponi, A. J.: Mode of action of lecithin in suppressing cholesterol absorption. J. Lip. Res. 22: 744, 1981.

9. Friedman, G. D.: The relationship between coronary heart disease and gallbladder disease. A critical review. Ann. Int. Med. 68:222, 1968.

10. Friedman, G. D., and Kannel, W. B.: The epidemiology of gallbladder disease, observation in the Framingham study. J. Chron. Dis. 19:273, 1966.

11. Ahlberg, J., Angelin, B., Einarsson, K., Hellström, K., and Leijd, B.: Prevalence of gallbladder disease in hyperlipoproteinemia. Dig. Dis. Sci. 24:459, 1979.

12. Ahlberg, J.: Serum lipid levels and hyperlipoproteinaemia in gallstone patients. Acta Chir. Scand. 145: 373, 1979.

13. Einarsson, K., Hellström, K., and Kallner, M.: Gallbladder disease in hyperlipoproteinaemia. Lancet 1: 484, 1975.

14. Ahlberg, J.: Biliary lipid metabolism in hyperlipoproteinaemia and gallstone disease. Acta Chir. Scand. 492:1, 1979.

15. Angelin, B., Einarsson, K., and Leijd, B.: Biliary lipid composition during treatment with different hyperlipidaemic drugs. Europ. J. Clin. Invest. 9: 185, 1979.

16. Bateson, M. C., MacLean, D., Ross, P. E., and Bouchier, A. D.: Clofibrate therapy and gallstone induction. Dig. Dis. 23:623, 1978.

17. Goldstein, J. L., and Brown, M. S.: Atherosclerosis: The low-density lipoprotein receptor hypothesis. Metabolism 26:1257, 1977.

18. Fraser, G. E., Jacobs, D. R., Anderson, J. T., Foster, N., Palta, M., and Blackburn, H.: The effects of various vegetable supplements on serum cholesterol. Am. J. Clin. Nutr. 34:1272, 1981.

19. Schwartz, C. C., Halloran, L. G., Vlahcevic, Z. R., Gregory, D. H., and Swell, L.: Preferential utilization of free cholesterol from high-density lipoproteins for biliary cholesterol secretion in man. Science 200:62, 1978.

20. Dam, H.: Nutritional aspects of gallstone formation with particular reference to alimentary production of gallstones in laboratory animals. World Rev. Nutr. & Diet. 11:199, 1969.

21. Hikasa, Y., Maruyama, I., Yoshinaga, M., Hirano, M., Eguchi, T., Shioda, R., Tanimura, H., and Hashimoto, K.: Initiating factors of gallstones, especially cholesterol stones. Arch. Jap. Chir. 33:601, 1964.

22. Hikasa, Y., Kuyama, T., Maruyama, I., Yoshinaga, M., Hirano, M., Eguchi, T., Shioda, R., Tanimura, H., Hashimoto, K., Muroya, H., and Togo, M. Initiating factors of gallstones, especially cholesterol stones (II). Arch. Jap. Chir. 34:1430, 1965.

23. Høstmark, A. T., Spydevold, Ø., Lystad, E., and Eilertsen, E.: Plasma lipoproteins in rats fed starch, sucrose, glucose or fructose. Nutr. Rep. Int. 25:161, 1982.

24. Grundy, S. M.: Effects of PUFA's on lipid metabolism. J. Clin. Invest. 55:269, 1975.

25. Grundy, S. M.: Biliary lipids, gallstones and treatment of hyperlipidemia. Europ. J. Clin. Invest. 9:179, 1979.

26. Kritchevsky, D.: Vegetable protein and atherosclerosis. J. Am. Oil Chem. Soc. 56:135, 1979.

27. Sirtori, C. R., Agardi, E., Conti, F., Mantero, O., and Gatti, E.: Soybean-protein diet in the treatment of type-II hyperlipoproteinaemia. Lancet, 275, February 5, 1977.

28. Huff, M. W. and Carroll, K. K.: Effects of dietary protein on turnover, oxidation, and absorption of cholesterol, and on steroid excretion in rabbits. J. Lip. Res. 21:546, 1980.

29. Carroll, K. K.: Dietary protein in relation to plasma cholesterol levels and atherosclerosis. Nutr. Rev. 36:1, 1978.

30. Eklund, A., and Sjöblom, L.: Effects of the source of dietary protein on serum lower density lipoprotein (VLDL + LDL) and tocopherol levels in female rats. J. Nutr. 110:2321, 1980.

31. Sirtori, C. R., Gatti, E., Mantero, O., Conti, F., Agradi, E., Tremoli, E., Sirtori, M. T., Fraterrigo, L., Tavazzi, L., and Kritchevsky, D.: Clinical experience with the soybean protein diet in the treatment of hypercholesterolemia. Am. J. Clin, Nutr. 32:1645, 1979.

32. Kritchevsky, D., and Klurfeld, D. M.: Influence of vegetable protein on gallstone formation in hamsters. Am. J. Clin. Nutr. 32:2174, 1979.

33. Danielsson, H., and Sjövall, J.: Bile acid metabolism. Ann. Rev. Biochem. 44:233, 1975.

34. Montgomery, R. W., Dryer, R. A., Conway, T. W., and Spector, A. A.: Biochemistry. A case oriented approach. The C. V. Mosby Company, St. Louis, Missouri, 1980.

35. Martin, D. W., Mayes, P. A., and Rodwell, V. W.: Harper's Review of Biochemistry. Lange Medical Publications, Los Altos, California, 1981.

36. Cantafora, A., Angelico, M., DiBiase, A., Pieche, U., Bracci, F., Attili, A. F., and Capocaccia, L.: Structure of biliary phosphatidylcholine in cholesterol gallstone patients. Lipids 16:589, 1981.

37. Gurr, M. I., and James, A. T.: Lipid Biochemistry, an introduction. Chapman and Hall, New York, New York, 1980.

38. Wilson, J. D.: The role of bile acids in the overall regulation of steroid metabolism. Arch. Int. Med. 130:493, 1972.

39. Redinger, R. N., and Small, D. M.: Bile composition, bile salt metabolism and gallstones. Arch. Intern. Med. 130:618, 1972.

40. Fredrickson, D. S., Levy, R. I., and Lees, R. S.: Fat transport in lipoproteins. An integrated approach to mechanism and disorders. N. Eng. J. Med. 276:34, 1976.

41. Greco, A. V., Mingrone, G., and Passi, S.: Bile acid content of gallbladder bile and stones in type IIb and IV hyperlipoproteinemia. Clin. Chimica Acta 116:81, 1981.

42. Kadziolka, R., Nilsson, S., and Schersten, T.: Prevalence of hyperlipoproteinemia in men with gallstone disease. Scand. J. Gastroent. 12:353, 1977.

43. Boston University Medical Center: A report from the Boston Collaborative Drug Surveillance Program. N.E.J. Med. 290:15, 1974.

44. The Coronary Drug Research Group: Gallbladder disease as a side effect of drugs influencing lipid metabolism. N. E. J. Med. 296:1185, 1977.

45. Grundy, S. M., and Mok, H. Y.: Colestipol, clofibrate and phytosterols in combination therapy and hyperlipidemia. J. Lab. Clin. Med. 89:354, 1977.

46. Sodhi, H. S., and Kudchodkar, B. J.: Correlating metabolism of plasma and tissue cholesterol with that of plasma-lipoproteins. Lancet, 513, March 10, 1973.

47. Smith, L. C., Pownall, H. J., and Gotto, A. M.: The plasma lipoproteins:structure and metabolism. Ann. Rev. Biochem. 47:751, 1978.

48. Beher, W. T., Baker, G. D., and Penney, D. G.: Comparative study of the effects of bile acids and cholesterol on cholesterol metabolism in the mouse, rat, hamster and guinea pig. J. Nutr. 79:523, 1963.

49. Ho, K. J.: Comparative studies on the effect of cholesterol feeding on biliary composition. Am. J. Clin. Nutr. 29:698, 1976.

50. Tan, M. H., Dickinson, M. A., Albers, J. J., Havel, R. J., Cheung, M. C., and Vigne, J. L.: The effect of a high cholesterol and saturated fat diet on serum high-density lipoprotein-cholesterol, apoprotein A-1, and apoprotein E levels in normolipidemic humans. Am. J. Clin. Nutr. 33:2559, 1980.

51. Schaefer, E. J., Levy, R. I., Ernst, N. D., Sant, F. D., and Brewer, H. B.: The effects of low cholesterol, high polyunsaturated fat, and low fat diets on plasma lipid and lipoprotein cholesterol levels in normal and hypercholesterolemic subjects. Am. J. Clin. Nutr. 34:1758, 1981.

52. Shorey, R. L., Bazan, B., Lo, G. S., and Steinke, F. H.: Determinants of hypocholesterolemic response to soy and animal protein-based diets. Am. J. Clin. Nutr. 34:1769, 1981.

53. Christensen, F., Dam, H., and Prange, I.: Alimentary production of gallstones in hamsters. II. Acta Physio. Scand. 27:315, 1952.

54. Munoz, J., Sanstead, H. H., Jacob, R. A., Logan, G. M., Reck, S. J., Klevay, L. M., Dintzis, F. R., Inglett, G. E., and Shuey, W. C.: Effects of some cereal brans and textured proteins on plasma lipids. Am. J. Clin. Nutr. 32:580, 1979.

55. Heaton, K. W.: Aktuelle Probleme der Klinischen Dietetik. Dietary fiber in relation to cholesterol and bile acid metabolism and to the pathogenesis of gallstones. Kasper, H. Thieme, Stuttgart, Germany, 1978.

56. Sacks, F. M., Castelli, W. P., Donner, A., and Kass, E. H.: Plasma lipids and lipoproteins in vegetarians and controls. N. Eng. J. Med. 292:1148, 1975.

57. Carroll, K. K., and Hamilton, R. M. G.: Symposium: Nutritional perspective and atherosclerosis. Effects of dietary protein and carbohydrate on plasma cholesterol levels in relation to atherosclerosis. J. Food Sci. 40:18, 1975.

58. Forsythe, W. A., Miller, E. R., Hill, G. M., Romsos, D. R., and Simpson, R. C.: Effects of dietary protein and fat sources on plasma cholesterol parameters, LCAT activity and amino acid levels and on tissue lipid content of growing pigs. J. Nutr. 110:2467, 1980.

59. Roy, D. M., and Schneeman, O. B.: Effect of soy protein, casein and trypsin inhibitor on cholesterol, bile acids and pancreatic enzymes in mice. J. Nutr. 111:878, 1981.

60. van Raaij, J. M. A., Katan, M. B., Hautvast, J. G., and Hermus, R. J.: Effects of casein versus soy protein diets on serum cholesterol and lipoproteins in young healthy volunteers. Am. J. Clin. Nutr. 34: 1261, 1981.

61. Huff, M. W., and Carroll, K. K.: Effects of dietary proteins and amino acid mixtures on plasma cholesterol levels in rabbits. J. Nutr. 110:1676, 1980.

62. Park, M., and Liepa, G. U.: Effects of dietary protein and amino acids on the metabolism of cholesterol-carrying lipoprotein in rats. J. Nutr. 112:1892, 1982.

63. Ginter, E.: Cholesterol: Vitamin C controls its transformation to bile acid. Science 179:702, 1972.

64. Gruberg, E. R., and Raymond, S. A.: Beyond cholesterol. A new theory on arteriosclerosis. Atlantic Monthly, 59, May, 1979.

65. Danzinger, R. G., Hofmann, A. F., Thistle, J. L., and Schoenfield, L. J.: Effect of oral chenodeoxycholic acid on bile acid kinetics and biliary lipid composition in women with cholelithiasis. J. Clin. Invest. 52:2809, 1973.

66. Reynier, M. O., Montet, J. C., Gerolami, A., Marteau, C., Crotte, C., Montet, A. M., and Mathieu, S.: Comparative effects of cholic, chenodeoxycholic and ursodeoxycholic acids on micellar solubilization and intestinal absorption of cholesterol. J. Lip. Res. 22:467, 1981.

67. Kubota, S., Kajiyama, G., Sasaki, H., Kawamoto, T., and Miyoshi, A.: Lipid metabolism in the development of cholesterol gallstones in hamsters. II. The effect of dietary cholesterol on biliary phospholipids and gallstone formation. Hiroshima J. Med. Sci. 29: 143, 1980.

68. Einarsson, K., Hellström, K., and Kallner, M.: Bile acid kinetics in relation to sex, serum lipids, body weight and gallbladder disease in patients with various types of hyperlipoproteinemia. J. Clin. Invest. 54:1301, 1974.

69. Mok, H. Y., Von Bergman, K., Crouse, J. R., and Grundy, S. M.: Biliary lipid metabolism in obesity. Effects of bile acid feeding before and during weight reduction. Gastroent. 76:556, 1979.

70. Grundy, S. M.: Treatment of hypercholesterolemia by interference with bile acid metabolism. Arch. Int. Med. 130:638, 1972.

71. Schlierf, G., Schellenberg, B., Stiehl, A., Czygan, P., and Oster, P.: Biliary cholesterol saturation and weight reduction-effects of fasting and low calorie diet. Digestion 21:44, 1981.

72. Lawrie, R. A.: Proteins as Human Food. The AVI Publishing Company, Westport, Connecticut, 1970.

73. Roeschlau, P., Bernt, E., and Gruber, W.: Enzymatic determination of total cholesterol in serum. Z. Klin. Chem. Klin. Biochem. 12:226, 1974.

74. Warnick, G. R., and Albers, J. J.: A comprehensive evaluation of the heparin-manganese precipitation procedure for estimating high density lipoprotein cholesterol. J. Lip. Res. 19:65, 1978.

75. Reyes, H., and Kern, F.: Effect of pregnancy on bile flow and biliary lipids in the hamster. Gastroent. 76:144, 1979.

76. Turley, S. D., and Dietschy, J. M.: Re-evaluation of the 3-alpha-hydroxysteroid dehydrogenase assay for total bile acids in bile. J. Lip. Res. 19:924, 1978.

77. Trudinger, P. A.: Determination of lipid phosphorus in the nanomolar range. Short communications. Analyt. Biochem. 36:225, 1970.

78. Winer, B. J.: Statistical Principles in Experimental Design. McGraw-Hill, New York, New York, 1971.

79. Mahfouz, S.: Comparative effect of dietary animal and vegetable protein on cholesterol gallstone formation and biliary constituents in the hamster. Thesis, Texas Woman's University, May, 1981.

80. Kruski, A.: Personal communication. University of Texas Health Science Center, San Antonio, Texas, 1982.