STUDY ON THE EFFECT OF 2,6-DIHYDROXYACETOPHENONE ON CHOLESTEROL METABOLISM IN HYPERLIPIDEMIC HAMSTERS

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2,6-Dihydroxyacetophenone (2,6-DHA) is an analogue of 2,4,6-trihydroxyacetophenone (THA), aglycone part of naturally occurring glucoside, 4,6-dihydroxy-2-O- (β-D-glucopyranosyl) acetophenone found in *Curcuma comosa* Roxb. (Family *Zingiberaceae*). 2,4,6-THA has been reported to effectively stimulate bile secretion by enhancing bile acid excretion resulting in decreased plasma cholesterol. Attempts have been made in this study to find out the mechanism by which 2,6-dihydroxyacetophenone (DHA) lowers plasma cholesterol in hypercholesterolemic hamsters. Studies were performed in male hamsters induced to become hyperlipidemic by supplementing cholesterol 0.2% body weight (BW) in corn oil for 3 weeks. After plasma cholesterol was elevated to 250-350 mg/dl, a dose of 2,6-DHA (300-800 μ mol/kg body weight) and duration of treatment which could reduce plasma lipids were determined. It was found that the optimum dose of 2,6-DHA which could reduce plasma cholesterol to 46% and plasma triglyceride to 73% of the untreated group was 400 μmol/kg body weight and the suitable duration was 10 days. In order to determine how 2,6-DHA could reduce plasma cholesterol, livers of 2,6-DHA treated and untreated animals were analyzed for free cholesterol, cholesteryl ester and triglyceride contents. Liver microsomes were analyzed for the activity of cholesterol 7α-hydroxylase, and a regulatory enzyme for conversion of cholesterol to bile salt. In feces, bile salt and total cholesterol were determined and in plasma, the distribution of cholesterol in VLDL, LDL and HDL fractions were measured. In the 2,6-DHA treated group, there was no significant difference on liver free cholesterol, cholesteryl ester or triglyceride content when compared with those of the untreated group. This indicated that 2,6-DHA had no effect on cholesterol storage and the key enzyme involved in cholesteryl ester synthesis, was acyl CoA cholesterol acyltransferase. However, in the liver microsome the activity of cholesterol 7α-hydroxylase increased 7-fold in animals fed 2,6-DHA compared to that of untreated animals. In feces of treated animals the bile salt and total cholesterol increased 2- and 6-fold respectively. Moreover, regarding distribution of cholesterol in various fractions of plasma lipoproteins, 2,6-DHA decreased cholesterol in VLDL and LDL but not in HDL fraction.

The results obtained in this study indicate that the hypocholesterolemic effect of 2,6-DHA was on the activation of cholesterol 7α-hydroxylase activity and the excretion of cholesterol and bile salt in feces. The increased fecal excretion of bile salt would up-regulate LDL-receptors leading to a lowering of cholesterol in VLDL and LDL fraction. Accordingly, this compound may have potential for development as a therapeutic agent for treatment of cholestasis, dissolving gallstones and lowering plasma lipid.

4036427 SCBC/M: สาขาวิชา : ชีวเคมี; วท. ม. (ชีวเคมี)

ศิริพร ถนอมชาติ : การศึกษาผลของ 2,6 ไดไฮดรอกซีอะซีโตฟีโนน ต่อเมตาบอลิสมของโคเลสเตอรอลในหนูแฮมสเตอร์ที่มีไขมันสูง (STUDY ON THE EFFECT OF 2,6-DIHYDROXYACETOPHENONE ON CHOLESTEROL METABOLISM IN HYPERLIPIDEMIC HAMSTERS) คณะกรรมการควบคุมวิทยานิพนธ์: ประหยัด โกมารทัต, Ph. D., ภาวิณี ปิยะจตุรวัฒน์ Ph. D., ประพนธ์ วิไลรัตน์, Ph. D. 125 หน้า. ISBN 974-664-177-8.

2,6-Dihydroxyacetophenone (DHA) เป็นอนุพันธ์ของ 2,4,6-trihydroxyacetophenone (THA) ซึ่งเป็นส่วนที่ไม่ใช่น้ำตาล (aglycone) ของไกลโคไซด์ 4,6-dihydroxy-2-O- (β-D-glucopyranosyl) acetophenone ที่มีอยู่ในพืชสมุนไพรว่านชักมดลูก ซึ่งเป็นพืชในตระกูล *Zingiberaceae* ได้มีรายงานว่า 2,4,6-THA มีฤทธิ์ในการกระตุ้นการหลั่งน้ำดี โดยการเพิ่มการขับออกของเกลือน้ำดี เป็นผลทำให้สามารถลดระดับโคเลสเตอรอลในเลือดได้ งานวิจัยนี้มีจุดประสงค์ที่จะศึกษากลไกลการทำงานของ 2,6-DHA ในการลดระดับโคเลสเตอรอลในเลือดของแฮมสเตอร์โดยทำการทดลองในหนูแฮมสเตอร์เพศผู้ซึ่งถูกเหนี่ยวนำให้อยู่ในสภาวะที่มีระดับโคเลสเตอรอลในเลือดสูง ประมาณ 250-350 mg/dl ด้วยการป้อนโคเลสเตอรอล 0.2% น้ำหนักตัว ที่ละลายในน้ำมันข้าวโพด เป็นเวลา 3 สัปดาห์ จากนั้นได้ทำการให้ 2,6-DHA (300-800 μmol/kg body weight) ที่ระยะเวลาต่างๆกัน เพื่อหาปริมาณและระยะเวลาของการให้ 2,6-DHA ที่เหมาะสมที่สามารถลดระดับไขมันในพลาสมาได้ ผลการทดลองพบว่า ปริมาณของ 2,6-DHA ที่ต่ำที่สุดที่สามารถลดระดับพลาสมาโคเลสเตอรอลได้ 46% และไตรกลีเซอไรด์ได้ 73% คือ 400 μmol/kg body weight และระยะเวลาที่เหมาะสมในการให้คือ 10 วัน ดังนั้นจึงได้ทำการศึกษากลไกลการทำงานของ 2,6-DHA ในการลดระดับโคเลสเตอรอลในพลาสมา โดยการวัดระดับโคเลสเตอรอลรูปอิสระ รูปเอสเตอร์ ไตรกลีเซอไรด์ในตับและ activity ของเอนไซม์ cholesterol 7α-hydroxylase ใน liver microsomes ซึ่งเป็นเอนไซม์ที่ควบคุมการเปลี่ยนโคเลสเตอรอลในตับให้เป็นเกลือน้ำดีรวมทั้งระดับของโคเลสเตอรอลในพลาสมาไลโปโปรตีน(VLDL, LDL และ HDL) ผลการทดลองที่ได้พบว่าในแฮมสเตอร์ที่ให้และไม่ได้ให้ 2,6-DHA ปริมาณโคเลสเตอรอล, โคเลสเตอรอลเอสเตอร์ และ ไตรกลีเซอไรด์ในตับ จะมีปริมาณไม่แตกต่างกันอย่างมีนัยสำคัญ แสดงให้เห็นว่า 2,6-DHA ไม่มีผลต่อเอนไซม์ Acyl CoA: cholesterol acyltransferase (ACAT) และไม่ทำให้มีการสะสมไขมันในตับ แต่ได้พบว่าในกลุ่มที่ได้รับ 2,6-DHA activity ของ เอนไซม์ cholesterol 7 α-hydroxylase เพิ่มขึ้น 7 เท่า และมีปริมาณของ bile salt และโคเลสเตอรอลในอุจจาระเพิ่มขึ้น นอกจากนี้ยังพบว่า 2,6-DHA มีผลในการลดโคเลสเตอรอล VLDL และ LDL แต่ HDL ไม่เปลี่ยนแปลง จากผลการทดลองสรุปได้ว่า 2,6-DHA สามารถลดระดับโคเลสเตอรอลในเลือดของแฮมสเตอร์ที่อยู่ในสภาวะ hyperlipidemia คล้ายคลึงกับ 2,4,6-THAโดยการเพิ่ม activity ของเอนไซม์ cholesterol 7 α-hydroxylase ทำให้โคเลสเตอรอลในตับถูกเปลี่ยนให้เป็น bile salt มากขึ้นและเพิ่มการขับออกของ เกลือน้ำดี และโคเลสเตอรอลทางอุจจาระ นอกจากนี้ 2,6-DHA ยังมีผลทำให้มีการลดปริมาณโคเลสเตอรอลใน ไลโปโปรตีน VLDL และ LDL ทำให้ลดอัตราการเสี่ยงต่อการเกิดโรคหลอดเลือดอุดตัน ในงานวิจัยเรื่องนี้ให้แนวทางในการที่จะพัฒนานำ 2,6-DHAไปใช้เป็นยาลดระดับโคเลสเตอรอลและไขมันในเลือด และในขณะเดียวกันอาจจะช่วยป้องกันการเกิดนิ่วในถุงน้ำดี

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LIST OF ABBREVIATIONS

2,6 DHA	= 2,6-dihydroxyacetophenone
ACAT	= Acetyl-CoA: cholesterol acyltransferase
C.comosa	= *Curcuma comosa*
EtOH	= Ethanol
HDL	= High density lipoprotein
HMG-CoA	= 3-Hydroxy-3-methylglutaryl-coenzyme A
LCAT	= Lecithin cholesterol acyltranferase
LDL	= Low density lipoprotein
LPL	= Lipoprotein lipase
IDL	= Intermediate density lipoprotein
Mw	= Molecular weight
PPO	= 2,5-Diphenyloxazole
POPOP	= [1,4-Bis (2-5 Phenyloxazolyl)] benzene
SDS	= Sodium dodecyl sulfate
SEM	= Standard error of mean
TLC	= Thin layer chromatography
Tris	= Tris (hydroxymethyl) aminomethane
VLDL	= Very low density lipoprotein
$\bar{X}$	= Mean
g	= Gram
kg	= Kilogram
µl	= Microliter

LIST OF ABBREVIATIONS (CON.)

µM	= Micromolar
mg	= Milligram
ml	= Milliliter
Mm	= Millimolar
µmole	= Micromolar

CHAPTER I

INTRODUCTION

Coronary heart disease, particular atherosclerosis is the common cause of mortality. The risk factors including hyperlipidemia, hypertension and cigarette smoking (1). Hyperlipidemia is an important risk factor for development of heart disease especially high risk is observed in the presence of elevated plasma triglyceride and cholesterol levels. In addition, high levels of LDL-cholesterol and low HDL-cholesterol are markers of the increased risk (1). Several methods including dietary control and excerise have been employed to lower plasma lipids but they are successful only in some patients. Therefore treatment by drug therapy are still needed. Recently, the biological activity of an indigenous plant, *Curcuma comosa* Roxb. Family *Zingiberaceae*, which has been traditionally used for treatment of postpartum uterine inflammation, and as a stomachic and cholagogue in Thailand has been investigated. A hexane extract of the plant was found to exhibit estrogenic-like activity (2,3), whereas an ethylacetate extract exhibited choleretic (4) and hypolipidemic active (5,6). One of the active components which has choleretic activity has been identified as phloracetophenone glucoside (5) in which its aglycone part of the compound was 2,4,6-trihydroxyacetophenone (THA). This compound exhibited choleretic activity by stimulating bile flow, increase bile acid excretion and lowering lithogenicity of bile (6). Bile acids are synthesized from cholesterol in liver with the enzyme cholesterol 7α-hydroxylase as the rate-limiting step in their synthesis. The excretion of bile acid is the major route for removal of cholesterol from the body. Therefore any compound that

can stimulate bile acid excretion may have potential candidate compound to reduce plasma cholesterol. In this regard, the effect of 2,4,6-THA on increasing bile flow and bile acid excretion made this compound is of interest in having potential to be developed as a therapeutic agent in lowering plasma cholesterol. In addition to 2,4,6-THA, several analogues of acetophenone including 2,6-dihydroxyacetophenone (DHA) have been shown to have choleretic activity and biliary secretion of bile acid. It was also found that hydroxy substitution at 2-and 6 positions of the benzene ring in acetophenone were essential for the induction of high and output of bile. In the present study, 2,6-DHA was tested for its potency to be an hypolipidemic agent in comparison with 2,4,6-THA, which was carried out parallel in other study (6). The mechanisms by which 2,6-DHA and 2,4,6-THA act in lowering plasma cholesterol were also investigated.

The liver plays a central role in both the maintenance of whole body cholesterol homeostasis and the regulation of plasma lipoprotein cholesterol concentrations. The level of cholesterol in plasma usually reflects the balance between cholesterol input and output. The two input pathways include de novo cholesterol synthesis and uptake of dietary cholesterol whereas conversion of hepatic cholesterol to bile acids and biliary excretion of cholesterol are the only significant output pathways. In response to changes in cholesterol input or output, cholesterol balance across the liver cells in maintained by altering the flux of cholesterol through 1) endogenous cholesterol synthesis, 2) lipoprotein uptake, synthesis and secretion, 3) conversion of cholesterol to bile acids and 4) reversible conversion of excess cholesterol to cholesteryl esters. While hepatic free cholesterol and cholesteryl esters are maintained is dynamic

equilibrium, a regulated flux of cholesterol through these hepatic pools not only maintains free cholesterol levels within the hepatocyte but also influences the secretion of cholesteryl esters as components of plasma lipoproteins. The liver secretes cholesterol into the blood circulation together with triglycerides in the form of VLDL. VLDL is hydrolysed by endothelial lipoprotein lipase to form LDL which is removed from the circulation via both receptor and non-receptor dependent pathways located in liver and other peripheral tissues. An elevated concentration of LDL in plasma is an important risk factor for the development and progression of atherosclerotic cardiovascular disease (1).

Cholesterol 7α-hydroxylase is the key enzyme in the conversion of free cholesterol to liver into bile acids (7), which is subsequently excretion into bile. The activity of cholesterol 7α-hydroxylase activity appears to be regulated principally at the level of gene expression in response to bile acids fluxing through the liver in the enterohepatic circulation (7). Bile acid sequestrants such as cholestyramine binds bile acids in the intestinal lumen, thereby preventing their reabsorption. Loss of bile acids from the enterohepatic circulation results in derepression of 7α-hydroxylase expression and an increase in the rate of bile acid synthesis (7). The liver compensates for the accelerated loss of cholesterol by increasing the receptor dependent uptake of LDL from plasma (7), thereby lowering circulating LDL levels.

Golden hamsters are frequently called Syrian, in recognition of the country in which they were first discovered. A typical golden hamster is about 12.5 to 65 cm in length and 110 to 140 g weight. Hamsters are active in the evening and early morning, and possibly throughout the night. Breeding usually occurs in the spring and summer.

In hamster, cholesterol metabolism more closely resembles to that in man (91). For examples, the rate of total body and hepatic cholesterol synthesis are relatively low in both of these species when is expressed per kg of body weight and equal only about 1/10 the rates found in rat (91). In addition, the hamster, like man, carries much of its plasma cholesterol in LDL, responds to cholestyramine feeding with a fall in the concentration of plasma cholesterol, secretes bile relatively saturated with sterol, and develops cholesterol gallstones under certain dietary manipulations (91). Finally, receptor dependent and receptor independent mechanisms for the degradation of lipoproteins are important in both species for turnover of plasma LDL.

1. Cholesterol metabolism and its regulation

The metabolism of cholesterol has been a subject of great interest for many years. Cholesterol is the most abundant sterol in body and performs a number of essential functions for normal cells such as is a component of all cell membranes and function as precursor of bile acids, steroid hormones and vitamin D. It is of critical importance that the cell of major tissues of the body assured a continuous supply of cholesterol. To meet this need, a complex series of transport, biosynthetic and regulatory mechanisms has involved (8). However, it is also the major component of artherosclerotic plaques mainly in coronary arteries (9,10) and some type of gallstone in bile (11-13), therefore the regulation of cholesterol homeostasis in cell appears to be vital for proper cellular function.

Cholesterol in body comes from two sources, one is from dietary cholesterol which is exogenous source, the other are from synthesized by extra hepatic tissues and *de novo* synthesis of cholesterol by liver itself which are endogenous source. The liver

plays a central role in cholesterol synthesis (14). In addition, intestine, adrenal cortex, testis, skin, aorta and ovaries can also synthesize cholesterol (15-17). It is synthesized from acetate, which may come from catabolism of fatty acid, carbohydrate or some amino acid (18).

1.1. The endogenous pathway of cholesterol metabolism

The endogenous biosynthesis of cholesterol occurs in cytoplasm and endoplasmic reticulum. There are approximately 26 separate enzymatic reactions involved in biochemical conversion of acetate to complete the cholesterol structure, which may be divided in to 5 steps as follows.

a. Mevalonate, a-6-carbon compound is synthesized from acetyl CoA by three molecules of acetyl CoA combine in two successive reactions to form 3-hydroxy-3-methylglutaryl CoA (HMG-CoA). The enzyme HMG-CoA reductase catalyzes the formation of mevalonate (Figure A).
b. Isoprenoid units are formed from mevalonate by loss of CO_2.
c. Six isoprenoid units condense to form the intermediate, squalene.
d. Squalene cyclizes to give rise parent steroid, lanosterol.
e. Cholesterol is formed from lanosterol after several further steps, including the loss of 3 methyl groups. (Figure B).

The rate-limiting step in biosynthesis of cholesterol is reduction of HMG-CoA to mevalonic acid catalyzed by 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase (19-21,32). In a series of studies, Shapiro and Rodwell (22) have demonstrated that the alterations in HMG-CoA reductase activity measured in isolated microsomes closely parallel alteration in the intracellular rate of cholesterol synthesis

from acetate. That is, HMG-CoA reductase appears to be the rate regulatory site between acetyl CoA and cholesterol. Various factors can dramatically alter HMG-CoA reductase activity. Fasting (23), dietary cholesterol (22-25), glucagon or glucocorticoids (16) reduced reductase activity while injection of Triton (26-28), cholestryramine (28,29), tyroxine (23,30), insulin or thyroid hormone (16) increased the activity. HMG-CoA reductase exists in both active and inactive forms that may be reversible modified by phosphorylation-dephosphorylation mechanism (16), some of which may be cAMP dependent (16).

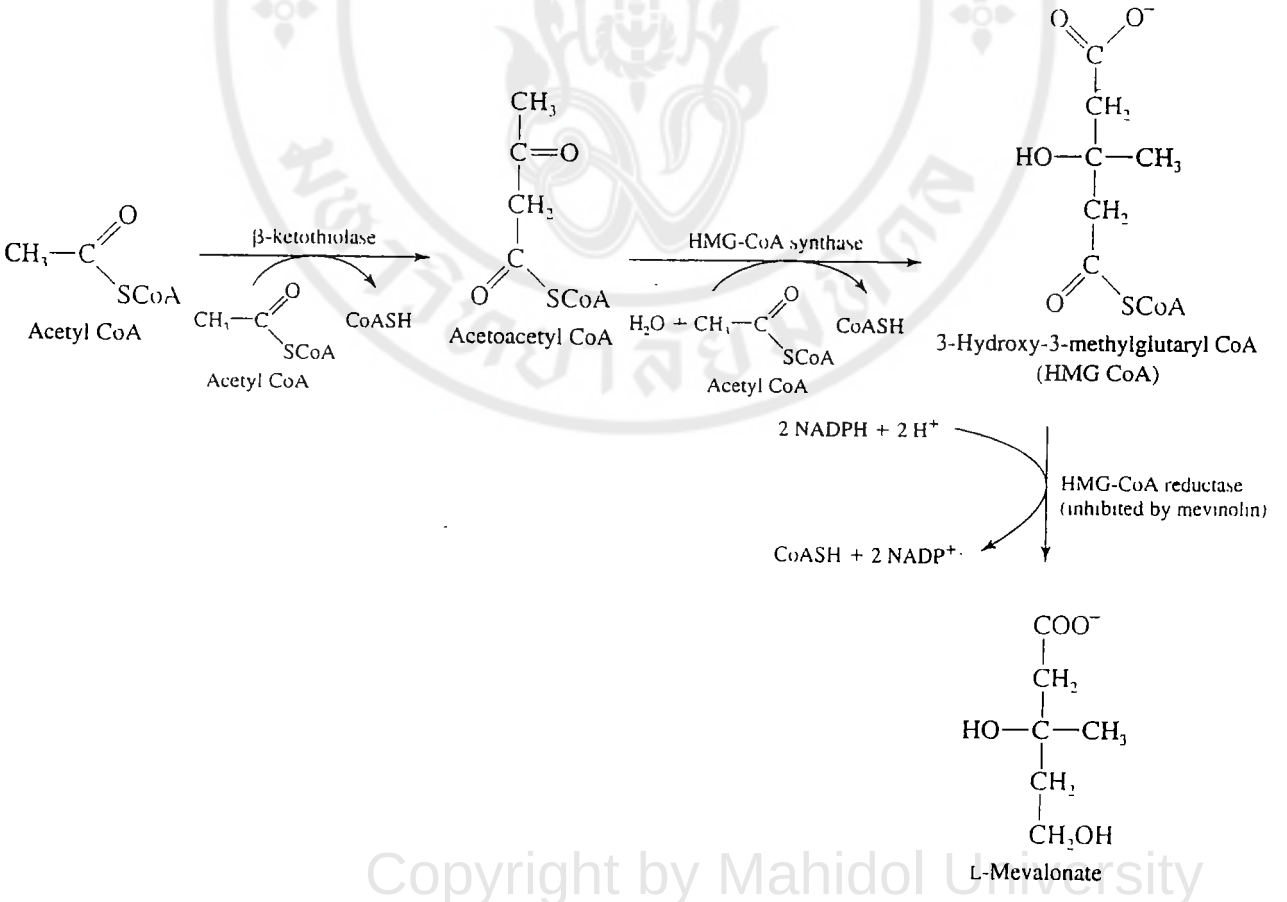


Figure A: The first step in biosynthesis of cholesterol, acetyl CoA to mevalonate (16).

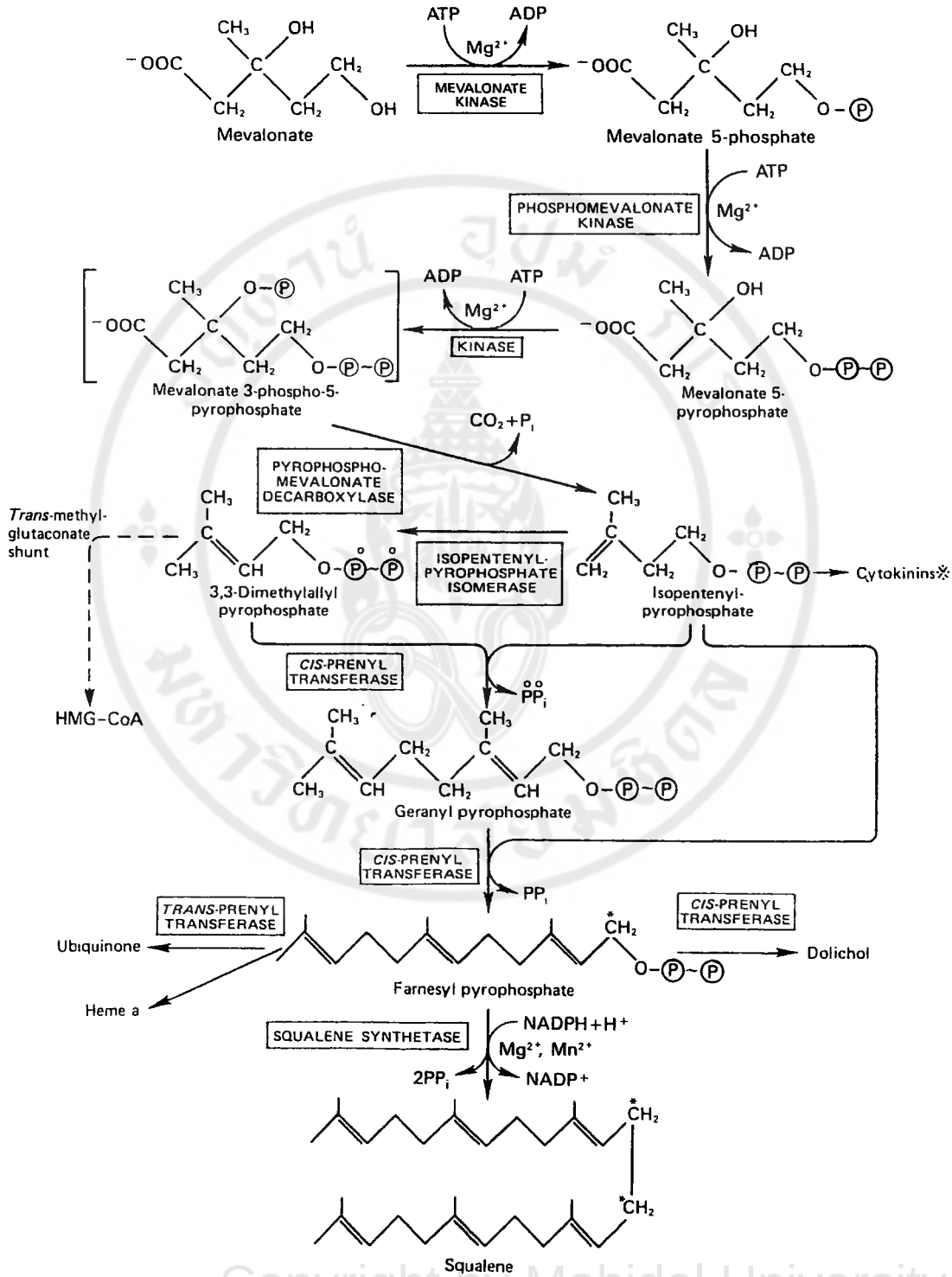


Figure B: Biosynthetic pathway of cholesterol (16).

On the catabolism of cholesterol, there are several pathways for elimination of cholesterol from the liver as bile acid, which is the major pathway, as unmodified cholesterol in bile, as component of plasma lipoproteins sent to the peripheral tissue or as bile salt secreted into the intestinal lumen. Continuous conversions of cholesterol into bile acid in the liver prevent the body from overload with cholesterol (31). The biosynthetic pathway of bile acid from cholesterol is devided into two parts (Figure C). The primary pathway for bile acid synthesis involved 7α –hydroxylation of cholesterol. Oxysterol 7α–hydroxylase participates in a secondary pathway, in which cholesterol is sequentially hydroxylated by mitochondrial cholesterol 27-hydroxylase, then by microsomal oxysterol 7α–hydroxylase. The two pathways which each utilize a different sterol 7α–hydroxylase produce distinct set of bile acid. The presence of both enzymes therefore generates a broad spectrum of bile acids, which allows the absorption of dietary fats (33).

The 7α–hydroxylation of cholesterol is the first committed step in the biosynthesis of bile acid, and this reaction is the rate limiting in pathway for synthesis of bile acid (35,36). The reaction is catalyzed by enzyme cholesterol 7α–hydroxylase (cholesterol, NADPH: oxidoreductase; EC 1.14.13.17). The enzyme is generally located in the edoplasmic reticulum of hepatocytes (34).

Cholesterol 7α–hydroxylase is microsomal mixed function oxidase whose function require NADPH, molecular oxygen, cytochrome P-450 and NADP-cytochrome P-450 reductase. From experiments *in vivo*, newly synthesized cholesterol in liver, rather than that derived from the plasma, is preferred substrate for cholesterol 7α–hydroxylase (36).

Cholesterol 7α–hydroxylase has been shown to be regulated by a number of factors including hormones, drugs, dietary components, lymphatic drainage, a bile acid sequestrant and bilary fistula (36,37). Moreover diurnal rhythm may have regulated cholesterol 7α–hydroxylase activity by increase maximum during the night and falling during the day (11).

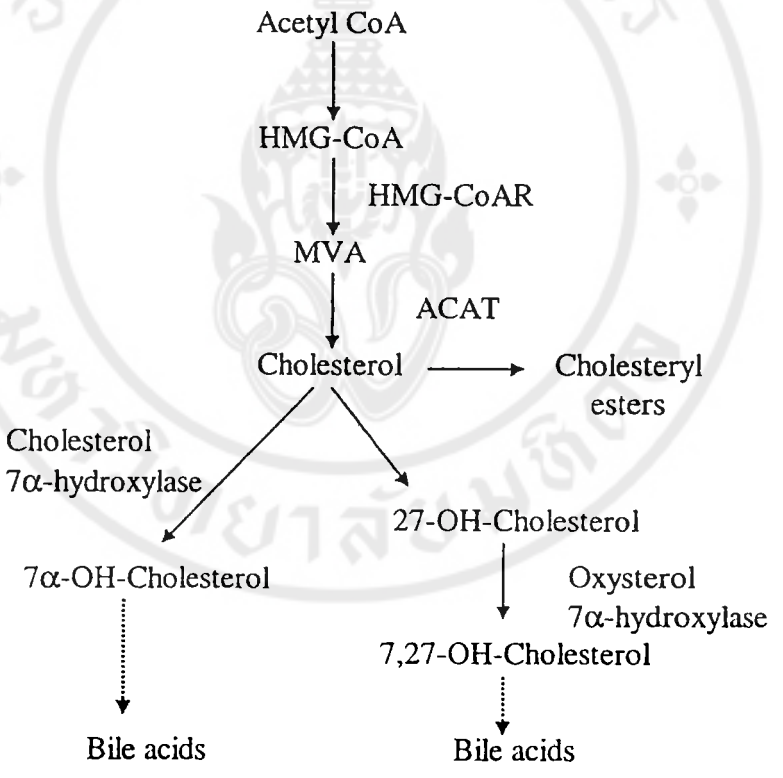


Figure C: Biosynthetic pathway of bile acid. HMG CoA, 3-hydroxy-3-methylglutaryl CoA; HMGCoAR, 3-hydroxy-3-methylglutaryl-CoA reductase; MVA, mevalonic acid; ACAT, acly-CoA cholesterol acyltransferase (33).

Two pathways of bile acid synthesized from cholesterol in the liver of most mammals are cholic and chenodeoxycholic acids. A proportion of primary bile acids reaching the lumen of the small intestine is modified by intestinal micro-organisms to form secondary bile acid lacking a 7α–hydroxy group namely deoxycholate arising from cholic acid and lithocholate from chenic acid (36). The bile acids normally enter the bile as glycine or taurine conjugated by amide link at C-24 to form glyco- or tauro-conjugated bile salts which more completely ionized and water-soluble than non-conjugated bile acid. The amide link of conjugated bile acid is resistant to hydrolysis by the pancreatic carboxypeptidase (14). Primary bile acids, which are synthesized from cholesterol in liver, are stored and concentrated in gallbladder when not needed immediately for digestion (34,39). When fat food is eaten, bile either from the liver or gallbladder are released into intestine via the cystic and common bile duct. Only a portion of the primary bile acid present in the intestine is converted to secondary bile acid through the action of bacterial enzyme (34). Both types of bile acids are reabsorbed from intestine to liver through portal vein and only small fraction is lost in feces as known as acidic sterols (34,38). The bile acids are removed from the portal blood by the liver, reconjugate with either glycine or taurine and then resecreted into the bile.

In rat, the secondary bile acids are hydroxylated in the 7α–position by hepatic enzymes and are then secreted into the intestine, together with reabsorbed and newly synthesized primary bile acid (36). Since reabsorption of bile acid from the intestinal lumen is incomplete, bile acids must be continually synthesized in the liver to balance the daily loss via the feces (36-38). The recycling of bile acids between intestine

and liver is called the enterohepatic circulation of bile acid as shown in (Figure E). Bile acid biosynthesis is regulated by the amount of cholesterol in liver and amount of bile acid that is returned from the intestine to liver (36,38) regulated bile acid biosynthesis.

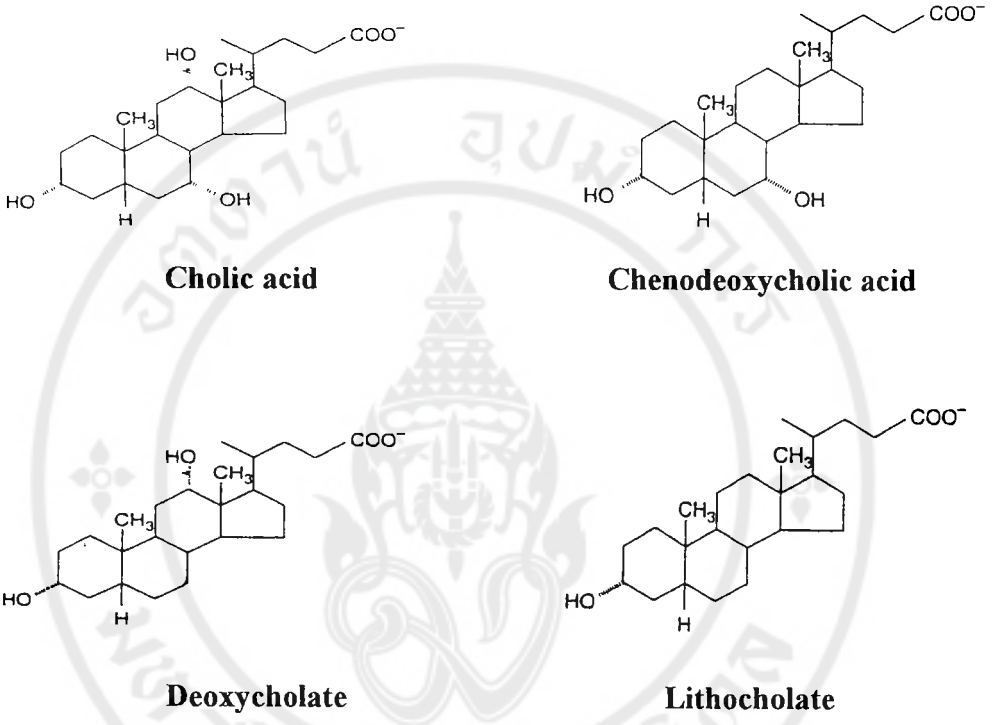


Figure D: Structure of primary (cholic acid, chenodeoxycholic acid) and secondary bile acid (deoxycholate, lithocholate) (8)

1.2 The exogenous pathway of cholesterol metabolism

The exogenous pathway of cholesterol metabolism has been described by Havel (40) and is shown in (Figure F). It begins in the intestine when cholesteryl ester in the diet is hydrolyzed to free cholesterol and combined with dietary free cholesterol and biliary cholesterol before being absorbed from the intestinal with other lipids. The mixed cholesterol is incorporated into chylomicrons, which enter to the blood stream. When chylomicron reacts with lipoprotein lipase to form chylomicron remnant, their

triglyceride is delivered to tissue for storage. The chylomicron remnant, containing cholesteryl esters is taken up by the liver when the remnant reacts with the apo-E receptor found only on liver.The cholesterol uptake may have four fates (a) reesterified and stored as cholesteryl ester, (b) secreted into plasma with lipoproteins, (c) converted into bile acid and (d) secreted into bile as cholesterol it self (41). The relative magnitudes of these pathways appear to be a function of the species and the metabolic state.

Liver secretes cholesterly ester into the blood circulation in the form of very-low density lipoprotein (VLDL). They are composed predominantly of triacylglycerol and cholesteryl esters and their function is to carry these lipids to peripheral tissues. VLDL has predominant protein namely apoprotein B-100 and E, both of which can be bound by LDL receptor (42). When VLDL particle reaches adipose tissue or muscle, it is converted to VLDL remnant upon removal of most triglyceride but retaining cholesteryl ester and two apoproteins (42). Liver takes up some VLDL remnants via LDL receptor on the surface of liver cell by endocytosis. Other VLDL remnants are further metabolized to yield a smaller particle namely low-density-lipoprotein (LDL). LDL particles retain apoB-100, but lose their other apoproteins. They contain much less triglyceride than their VLDL predecessors, and have a high concentration of cholesterol and cholesterol ester. ApoB-100 in LDL is a ligand for the LDL receptor and most LDL particles are eventually taken up into liver cell via LDL receptor (40,43). The affinity binding of LDL to LDL-receptor is much lower than of VLDL remnant, accounting for the longer lifespan of LDL in blood (normally 3 days) (43).

The amount of cholesterol liberated from LDL control the cell cholesterol metabolism (42). An accumulation of cholesterol modulates three processes as shown in figure G: (a) inhibition of endogenous cholesterol synthesis by turning off the function of HMG-CoA reductase, (b) increase synthesis of cholesteryl ester for storage by activating an enzyme acyl CoA: cholesterol acyltranferase (ACAT) (42,43). ACAT (EC 2.3.1.26) is located in the rough endoplasmic reticulum and esterifies excess intracellular cholesterol with long-chain fatty acid resulting cholesterol ester and stores in cytoplasm as lipid droplets (42,44) and (c) stop synthesizing new LDL receptor. Cells thereby adjust their receptors so that enough cholesterol is brought in to meet their varying demands but not enough to overload them.

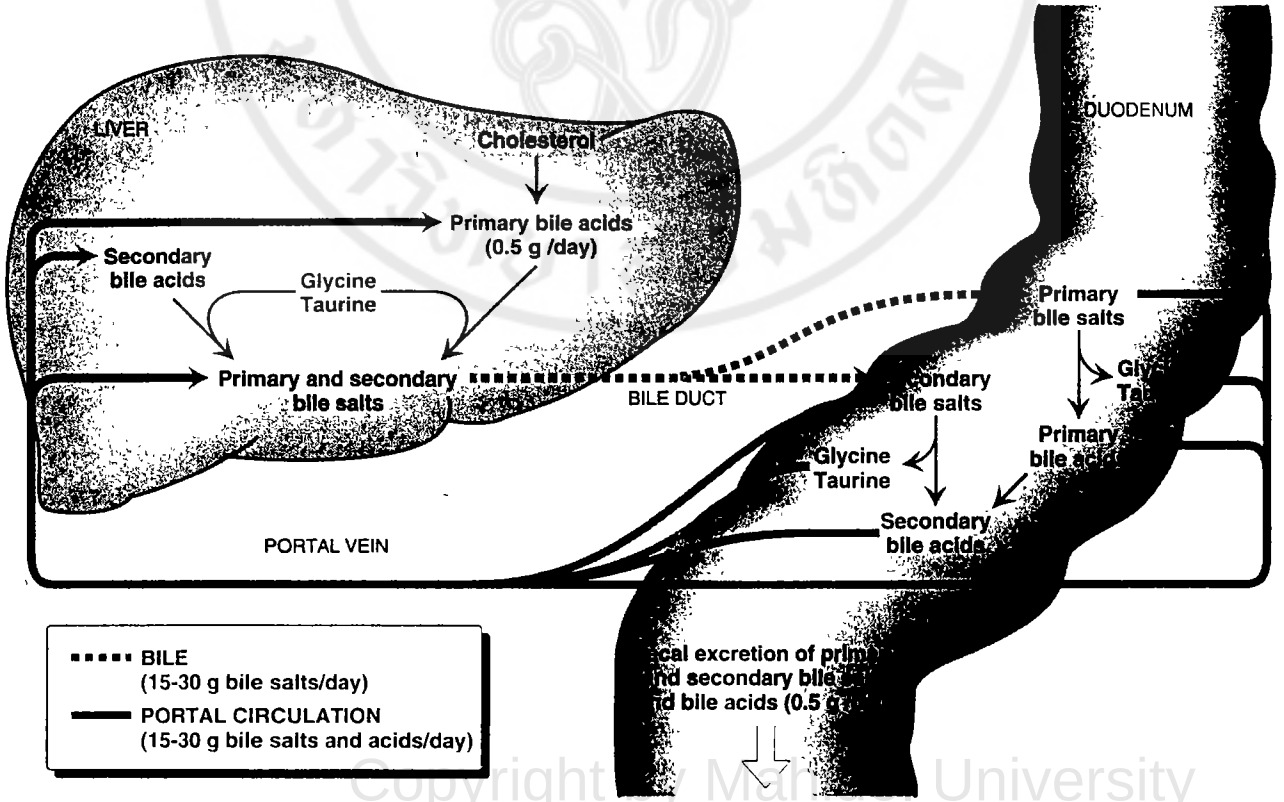


Figure E: Enterohepatic circulation of bile salt and bile acid (8).

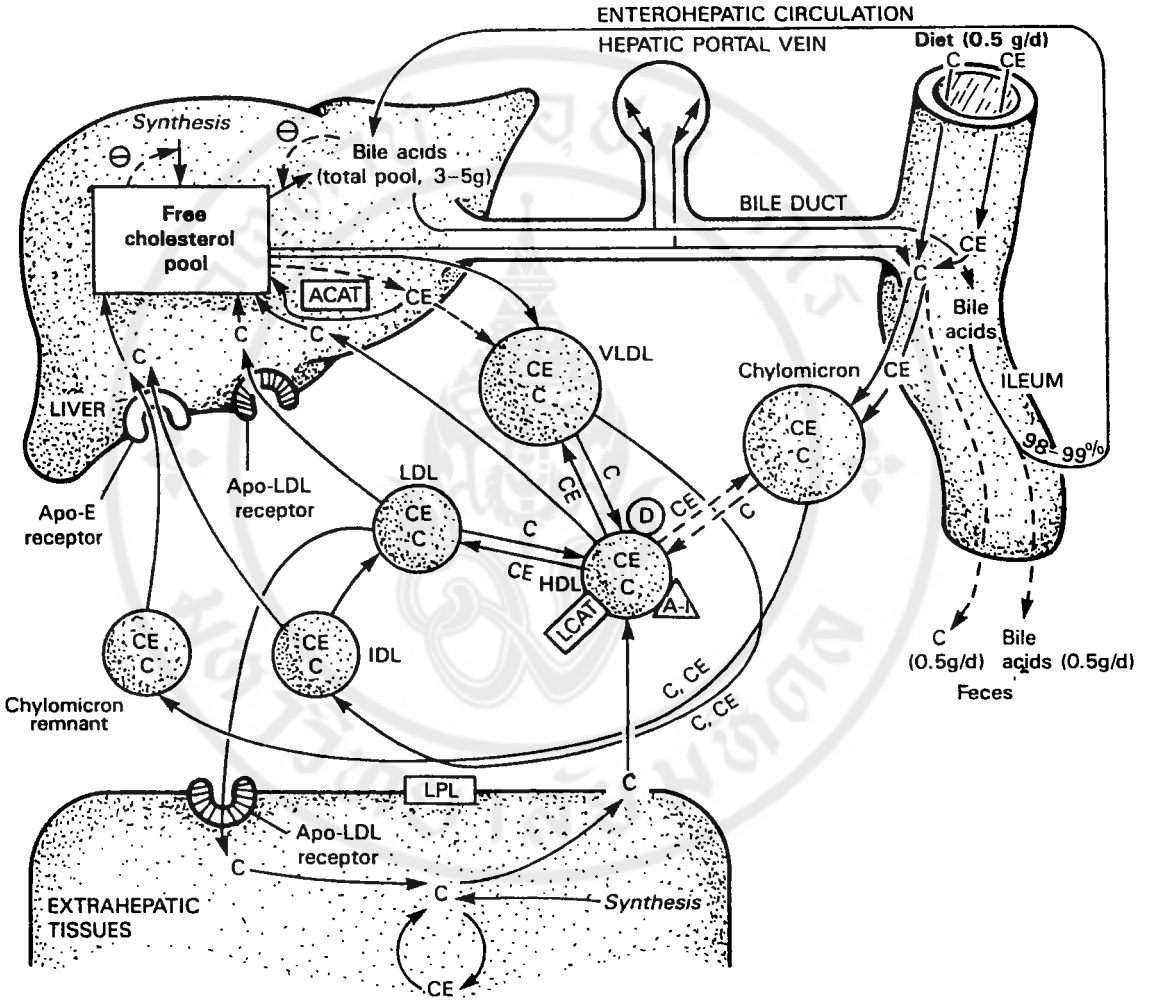


Figure F: Transport of cholesterol between tissue. C; free cholesterol, CE; cholesteryl ester, A-1; apoprotein A-1, D; cholesteryl ester transfer protein, LPL, lipoprotein lipase(16).

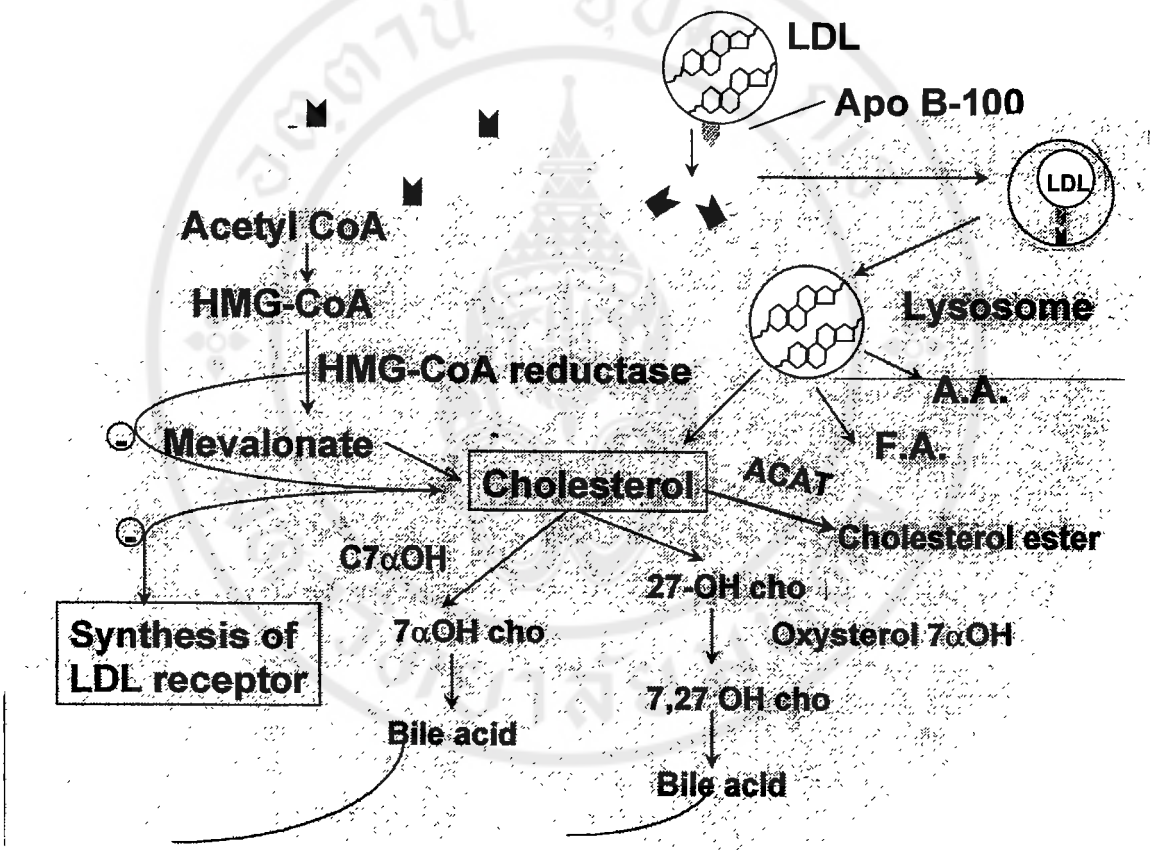


Figure G: The modulation of cholesterol in liver (8).

Another species of plasma lipoprotein namely high-density lipoprotein (HDL) functions to remove cholesterol from extrahepatic tissue to liver (42). The enzyme lecitin-cholesterol-acyltransferase (LCAT), a component in HDL by associated with a species of HDL containing apoA-I, esterifies cholesterol with a fatty acyl moiety derive from HDL lecitin, yielding both lysolecithin and cholesteryl ester. The actual substrate for LCAT is derived from the plasma membrane of cell which is transferred from extrahepatic cells to HDL. After esterification to VLDL and LDL it can be taken up by the liver via the LDL receptor (43). This pathway of cholesterol transport from extrahepatic cell to the liver is thought to be the major route of reverse cholesterol transport. Increasing evidence suggested that people who have higher HDL-cholesterol and low VLDL-and LDL-cholesterol have low risk to atherosclerosis (45-47).

2. Lipid lowering Agent

High levels of plasma lipids particularly cholesterol and triglyceride can be reduced by dietary method or by various drugs. In dietary modification, most hyperlipidemic patients find difficulty to maintain long term compliance with their prescribed diet. Therefore, agents that predominantly lower cholesterol and triglyceride have been used for reduction of plasma lipid concentration in hyperlipidemic patients. The hypolipidemic drugs are divided into two major groups as follows.

2.1 Cholesterol-Lowering Agent

2.1.1 HMG-CoA reductase inhibitor (Statin)

The HMG-CoA reductase inhibitors are considerable more active in reducing total cholesterol and LDL-cholesterol concentration in plasma than previously available drugs (48). Evidence is now available on their ability to improve survival in patients after myocardial an in those at high coronary risk but without established vascular disease. These finding provide the background for the rapid increase in the use of these agents.

The HMG-CoA reductase inhibitors currently in use include lovastatin (48), simvastatin (48), pravastatin (49) and fluvastatin (50). The mechanism of action of HMG-CoA reductase inhibitor act by inhibiting the enzyme responsible for catalyzing the conversion of 3-hydroxy-3-methylglutarly CoA to mevalonate, which is and early rate-limiting step in the biosynthesis of cholesterol. As a consequence of the inhibition of intracellular cholesterol synthesis, hepatocytes increase the expression of LDL receptor which then promote the extract of LDL-cholesterol from plasma. The drugs have no hypocholesterolemic activity in patients with homozygous familialr hypercholesterolaemia who lack gene necessary for the synthesis of LDL receptor (51).

The HMG-CoA reductase inhibitors differ substantially in their pharmaco-kinetic behaviors as that shown in Table A. Moreover, they appear to be equally effective, maximal doses reduce LDL-cholesterol by 30-35% (Table B), but the response is dose-dependent. They produce a lesser fall in plasma triglycerides (10 to 30%) by increasing the clearance of VLDL remnant by LDL receptors. The average HDL-cholesterol concentration is increased by 5 to 15% (51).

The adverse effect encountered with these agents are myositis and hepatotoxicity. Frank rhabdomyolysis occurs in only approximately 0 1% of patients receiving HMG-CoA reductase inhibitor monotherapy. The incidence is increased when these agents are used in combination with gemfibrozil, erythromycin or cyclosporin. Neither hepatic necrosis nor chronic liver disease has been reported on these agents (52).

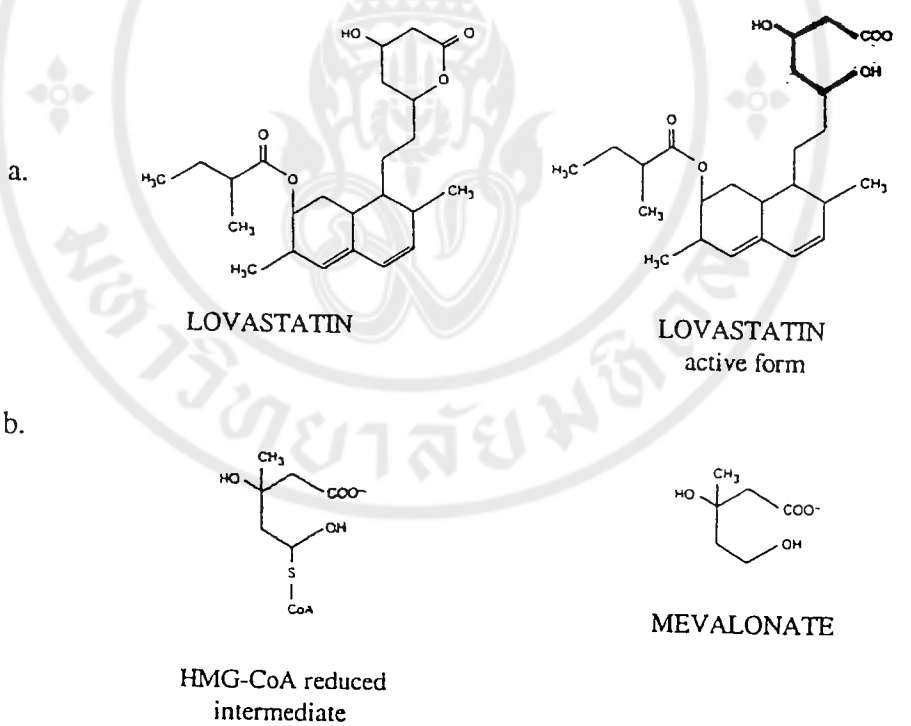


Figure H: HMG-CoA reductase inhibitor a: The structure of lovastatin and its active form showing the similarity to the normal HMG-CoA intermediate. b: HMG-CoA intermediate that immediate precursor a mevalonate.

Table A: Pharmacokinetic parameter of HMG-CoA reductase inhibitors (92).

Parameter	Fluvastatin	Lovastatin	Simvastatin	Pravastatin
Absorption (%)	98	30	60-85	35
Effect of food on absorption (% change in AUC)	↓15-25	↑50	0	↓30
Plasma protein binding (%)	≥99	≥95[a]	95-98[a]	45
Hepatic extraction (% of absorbed dose)	≥70	≥70	≥80	45
Crosses blood-brain barrier	No	Yes	Yes	No
Elimination half-life (h)	1.2	3	1.9[b]	3
Renal excretion (%)[c]	6	30	13	60

a For both parent drug and corresponding β-hydroxyacid metabolite.

b For main active metabolite.

c Renal excretion of radiolabelled parent drug plus metabolites is given as the percentage of an intravenous dose.

Abbreviation and symbols: AUC = area under the plasma concentration-time curve; ↓ = decreased; ↑ = increased.

2.1.2 Bile acid seguestrants

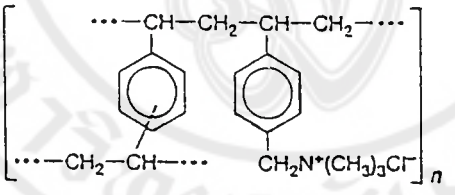
Cholestyramine and Cholestipol are insoluble anion exchange resins. After oral administration they release chloride ions and bind bile acid in their place. The resulting bile acid complex is not absorbed and are therefore largely excreted in the feces (53). The mechanism of action of bile acid sequestrants are interrupted enterohepatic circulation which lead to increase the synthesis of bile acid, using endogenous cholesterol as the substrate. The latter is derived partly from synthesis of cholesterol within liver cells and partly from the uptake of LDL-cholesterol from the plasma by increasing surface LDL receptor numbers.

Cholestyramine is formulated as powder. Sachetes of the powder contain 4 or 8 g of cholestyramine resin. The initial dose of 4 to 8 g daily is increased to a usual

maintenance level of 12 to 16 g daily. Cholestipol is formulated in 5 g sachets and its usual maintenance dose is 15 to 30 g daily.

The most significant drugs interactions associated with bile acid sequestrants result from their ability to bind fat-soluble drugs. This is clinically important when they are administered with drugs having a narrow therapeutic index such as anticoagulants, cardiac glucosides and thyroid hormones. To minimize the risk of interactions, other drugs should be given at least one hour prior to the resin but in the case of thyroxine, an interval of 4 to 5 hours is recommended (54).

a.



b.

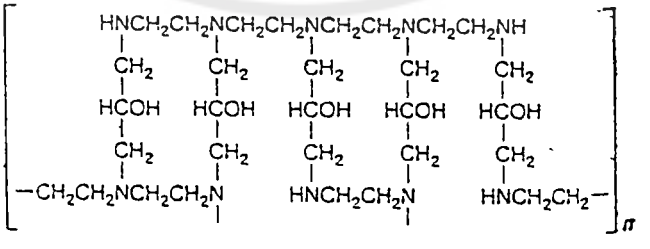


Figure I: Structure of bile acid sequestrants (a) cholestyramine, (b) cholestipol.

2.1.3 Probucol

Probucol is a lipid-lowering drug with strong antioxidant properties (55). When administered in dose of 500 mg twice daily, it reduces total cholesterol and LDL-cholesterol concentration by about 10 to 20% without substantially effecting serum VLDL-cholesterol or triglyceride levels. The mechanism of its lipid-lowering action is uncertained but may be related to increase catabolism of LDL-cholesterol (56).

Pharmacokinetic characteristics of Probucol is a lipid-soluble drug that concentrated in adipose tissue and is eliminated slowly with a half-life of between 20 and 50 days. During long term therapy, plasma drug concentrations rise slowly to plateau level after 3 to 4 months and are very variable among different individuals (56).

2.2 Triglyceride-Lowering Agent

2.2.1 Fibrates

The fibrates are a group of lipid lowering drugs structurally and functionally related to clofibrate. Clofibrate and gemfibrozil have been the most commonly used members of the group, although others such as fenofibrate, bezafibrate and ciprofibrate are also available. The major property of this group of drugs is their abilities to reduce plasma triglyceride concentrations by 20 to 30% .The reduction of total cholesterol concentrations is proportionally less (about 5 to 10%) while HDL-cholesterol concentrations generally increase by a similar proportion (57).

The mechanism of action of the fibrates affect several aspects of lipoprotein metabolism but it is not yet clear which is the principle effect and which are secondary effects (49,50). Lipoprotein lipase activity is increased and there is a marked

increase in the clearance of triglyceride from plasma. The increase in HDL-cholesterol is associated with an increase in the plasma concentration of HDL carrier apoproteins.

The dosages schedules of fibrates are shown in Table B and the adverse effect of the fibrates are generally well tolerated the most common immediate adverse effects such as abdominal discomfort, epgastric fullness, nausea and mild diarrhea. Moreover liver enzymes are sometimes slightly elevated and a syndrome of muscle tenderness with a raised serum creatine phosphokinase concentration has been observed, mainly in patients with the nephrotic syndrome.

Table B: Mechanism of action of lipid lowering drugs, dosage schedules and changes in lipid concentrations produced (93).

Group/mechanism of action	Drugs	Daily dosage	Lipid changes (%)			
			LDL-C	VLDL	HDL-C	Lp(a)
HMG CoA reductase inhibitors						
Inhibit intracellular cholesterol synthesis leading to an increase in hepatic LDL receptors. This results in increased clearance of cholesterol from plasma	Simvastatin Lovastatin Pravastatin Fluvastatin	10-40mg od 20-80mg od 10-40mg od 20-40mg od	↓30-35	↓10-30	↑5-15	No effect
Bile acid sequestrants						
Bind bile acids in the gut which interrupts their enterohepatic recycling. This leads to increased synthesis of new bile acids by hepatic uptake of circulating cholesterol	Cholestyramine Colestipol	4g bid to 8g tid 5g bid to 10g tid	↓10-30	↓0-15	No effect	No effect
Fibrates						
Major action uncertain. Increased lipoprotein lipax activity, reduced hepatic triglyceride synthesis and increased triglyceride clearance from plasma	Clofibrate Gemfibrozil Bezafibrate Fenofibrate	500mg tid - qid 600mg bid 200mg tid 100mg tid	↓5-10	↓20-30	↑5-10	No effect
Nicotinic acid (niacin) derivatives						
Reduce hepatic VLDL production	Nicotinic acid Acipimox	0.5-1.5g tid 250mg bid-tid	↓15-25[a]	↓15-40[a]	↑15-25[a]	↓5-10[a]

a Data for nicotinic acid.

Abbreviations and symbols: LDL-C = low-density lipoprotein cholesterol; HDL-C = high-density lipoprotein cholesterol; VLDL = very-low-density lipoprotein; od = once daily; bid = twice daily, tid = three times daily; qid = 4 times daily; ↓ = decreased; ↑ = increased.

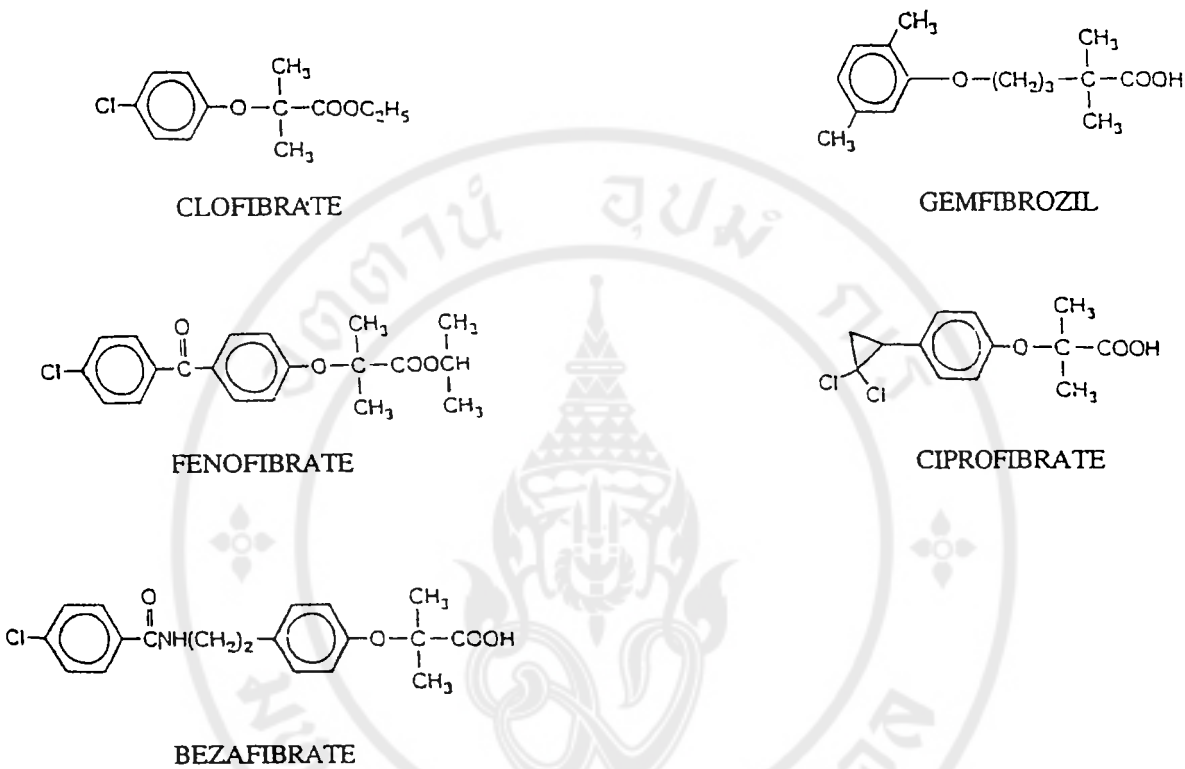


Figure J: Chemical structure of fibrate derivatives.

2.2.2 Nicotinic Acid (Niacin)

Nicotinic acid is a water-soluble B group vitamin which at high dose favorably affects all plasma lipid subtypes (58). It lowers plasma total and LDL-cholesterol concentrations, triglyceride and increase plasma HDL-cholesterol (59) An analogue of nicotinic acid, namely acipimox, which has a more prolonged effect is also available.

Nicotinic acid reduces VLDL production in the liver and since VLDL is converted to LDL, the LDL-cholesterol concentration also falls (60). A dose of 1.5 g

generally produces a 10 to 15% average reduction in LDL-cholesterol and a 15 to 25% reduction is produced by dose of 3 g. This dose level also reduce plasma triglyceride concentrations by 5 to 30%. The important adverse effect of nicotinic acid is hepatotoxicity (61).

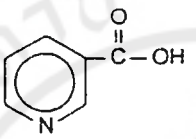


Figure K: Chemical structure of nicotinic acid.

3. *Curcuma comosa* Roxb. *(C. comosa)*

Curcuma comosa is a plant in *Curcuma* species of *Zingiberaceae* family. Member of this species, including *C. longa* Linn, *C. aromatica* Salisb, *C. zebloaria* Roxb., and *C. xanthorrhiza* Roxb. They have traditionally been used for medicinal purposes as anti- hepatotoxic (62), anti-inflammatory (63-65) emmenagogue (66) aromatic stomachics and bile-expelling agent (67,68). In 1993-1994, Yasni. *et al*, reported that *C. xanthorrhiza* exerts a marked influence in lowering triglyceride in male Sprague-Dawley rat (69) and α-curcumene was one of the active principle exerting triglyceride lowering activity in *C. xanthorrhiza* (70). *C. comosa* Roxb is commonly known as Waan Chak Mod Lok in Thai language, which has been widely used in folk medicine in Thailand. In the north-eastern part of Thailand, it is used for local application for inflammation of uterus. In the eastern region such as in Prachinburi province, it has been widely used to relieve the procidentia uteri and hemorrhoid. Studies on the

biological activities of the crude extract of *C. comosa*, indicated that the hexane extract was the most effective inducing uterotrophic (71) and estrogenic-like activities (72), whereas the butanol extract as well as the ethyl acetate extract exhibited the choleretic activity (73). One of the choleretic principles of the *C. comosa* rhizomes is a phloracetophenone glucoside [4,6-dihydroxy-2-O-(β-D-glucopyranosyl) acetophenone] (74). Evaluation on the choleretic activity of the glucoside indicated that the choleretic activity arise from the aglycone part of the compound which was phloracetophenone [2,4,6-trihydroxy acetophenone] (75).

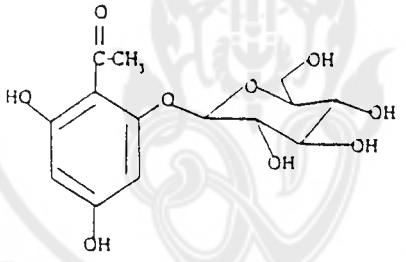


Figure L: Chemical structure of 4,6-dihydroxy-2-O-(β-D glucopyranosyl) acetophenone [phloracetophenone-2-O-(β-D glucopyranoside)]

2,6-Dihydroxy acetophenone (DHA) is an analog of phloracetophenone (2,4,6-THA) which has been demonstrated to increased bile flow rate, it also increase bile salt concentration but decreased biliary cholesterol, phospholipid and lowered bile lithogenic index (76). Moreover, this compounds greatly enhanced biliary excretory out put of cholesterol and bile acid which subsequently lower plasma cholesterol. Recently, the two compound are suggested to have potential for development as cholesterol lowering agent by in association with increase of total output of secreting bile acid and

bile cholesterol (77). Therefore, the compounds might be beneficial for therapeutic use for cholesterol gallstone dissolution and treatment of hyperlipidemia.

Figure M: Chemical structure of 2,4,6- trihydroxyacetophenone (2- acetylphloroglucinol, phloracetophenone) and 2,6-dihydroxyacetophenone

Hypolipidemic activity of other acetophenone derivatives has also been reported. In 1987-1988, Hall *et al* (78), demonstrated potent hypolipidimic activity of O-(N-phthalimido) acetophenone after administration over 8 weeks. The compound significantly reduced both serum cholesterol and triglyceride level in Sprague Dawly male rat (78) and modulated the lipid level by elevating high density lipoprotein cholesterol and reducing cholesterol content of very low density lipoprotein fraction.(78,79). The toxicity of this compound has not been reported.

This study aims to verify the hypocholesterolemic effect of 2,6-DHA in hypercholesterolemic hamsters induced by supplementing cholesterol at 0.2% body weight. The experiments were performed by feeding either placebo or 2,6-DHA dissolved in 10% ethanol and 90% corn oil to find out the optimum dose of 2,6-DHA in lowering plasma cholesterol and TG. The effect of 2,6-DHA on distribution of

cholesterol in plasma lipoproteins as well as total cholesterol, free cholesterol, cholesteryl ester and TG contents in liver were determined. Furthermore, in order to understand the effect of 2,6-DHA on cholesterol metabolism, the activity of hepatic cholesterol 7α-hydroxylase, a key enzyme in the conversion of cholesterol to bile acid was also determined. The excretion of biliary lipid especially total cholesterol and bile acid was also analyzed in feces too see whether 2,6-DHA had any effect on these biliary lipids excretion. The result of this experiment may suggest a therapeutic usefullness of 2,6-DHA in gallstone prevention.

The objectives of this study are

1. to investigate the time course on induction of hypercholesterolemia in male hamsters
2. to find out an optimum dose of 2,6-DHA in lowering plasma cholesterol and triglyceride concentration in hypercholesterolemic hamsters.
3. to determine the time course effect of 2,6-DHA at the optimum dose on plasma cholesterol and triglyceride concentration in hypercholesterolemic hamsters.
4. to find out the mechanism of action of 2,6-DHA in lipid metabolism
   a. determining the effect of 2,6-DHA on plasma lipoprotein-cholesterol distribution.
   b. determining the hepatic triglyceride and cholesterol contents.
   c. assaying the hepatic cholesterol 7α-hydroxylase activity.
   d. determining cholesterol and bile acid contents in feces.

CHAPTER II

MATERIALS AND METHODS

2.1 Chemicals

The chemical used in the present studies were obtained from the following companies.

Chemicals	Companies
- Albumin (bovine) crystal	- Calbiochem
- Cholesterol (5-Cholesten-3β-ol) for diet supplement	- Sigma Chemical Co. (St. Louis, Missouri, USA)
-[4-^{14}C]-cholesterol; specific activity 50 mCi/mmol	- Amercham-Searle, Alington Heights, Illinois,USA
- Corn oil (Mazola refined corn oil	- CPC Thailand Ltd.
- 2,6-Dihydroxyacetophenone	- Aldrich Chemical Company (St. Louis, Missouri, USA)
- DL-Dithiothereitol	- Sigma Chemical Co. (St. Louis, Missouri, USA)
- Enzymatic reagent kits for cholesterol, and triglyceride analysis	- BM Lab, Bangkok, Thailand
- Glucose-6-phosphate (monosodium salt)	- Sigma Chemical Co. (St. Louis, Missouri, USA.)

Chemicals	Companies
- Glucose-6-phosphate dehydrogenase (type IX) from Bakers Yeast	- Sigma Chemical Co. (St. Louis, Missouri, USA.)
- Iodine	- E. Merck (Darmstadt, Germany)
- β-Mercaptoethanol	- Sigma Chemical Co. (St. Louis, Missouri, USA.)
- β-Nicotinamide adenine dinucleotide phosphate (oxidised form, monosodium slat) from yeast	- Sigma Chemical Co. (St. Louis, Missouri, USA.)
- Organic solvents - benzene - chloroform - diethyl ether - ethanol (pharmaceutical grade) - ethyl acetate - isopropanol - methanol	- E. Merck (Darmstadt, Germany)
- Silica gel H (type60) for thin layer chromatography	- E. Merck (Darmstadt, Germnay)

2.2 Experimental animals

The adult male golden hamsters weighting between 80-120 g were obtained from the Animal Center, Faculty of Science, Mahidol University, Bangkok. They were housed in a room with temperature 25-30°C and kept under free access to chow diet

(C.P. Mice feed, Pokphan Animal Feed Co., Ltd. Yannawa, Bangkok, Thailand) and tap water *ad libitum*. The light cycle was alternated 12-hours period of light and dark (light on at 06.00 a.m.).

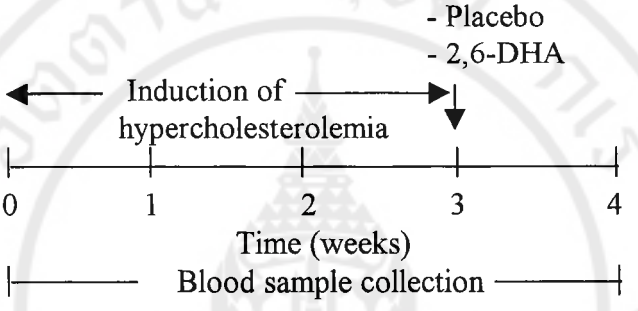
2.3 Induction of hypercholesterolemia in hamsters

All hamsters were divided into two groups: control and hypercholesterolemic groups. The control group was orally supplemented with corn oil 1 ml/day whereas the hypercholesterolemic group was supplemented with cholesterol in corn oil 20 g % at the dose of 0.2% BW (approximately 1 ml/day). Both groups were maintained on the chow diet and body weight of animals was recorded twice a week. During the feeding period, plasma cholesterol and triglyceride levels were followed by collecting the blood sample from orbital sinus under light ether anesthesia at 1, 2 and 3 week or otherwise indicated. About 3 weeks, plasma cholesterol in the cholesterol-fed hamsters became hypercholesterolemia with plasma cholesterol reach to the level approximately 250-350 mg/dl.

2.4 Treatment of hypercholesterolemic hamsters with 2,6-dihydroxy-acetophenone (DHA)

When plasma cholesterol of hamsters reached the level of 250-350 mg/dl, they were divided into two groups: treated and untreated with 2,6-DHA. The tested compound at various doses (300,400,600 and 800 μmol/kg BW) were primarily dissolved in absolute ethanol and further suspended with corn oil. In all cases, the amount of ethanol was limited to be less than 10% of total administered volume (0.5

ml). Equivalent amount of solvent (10% ethanol in corn oil) for dissolving 2,6-DHA was given to the untreated group. The hypercholesterolemic animals in treated and untreated group were orally feeding 2,6-DHA or its placebo solvent respectively at the total volume of 0.5 ml twice a day for one week as shown in the protocol below.



2.5 Collection of specimen

At the end of experiment, an overnight fasting animals were anesthetized between 8.00-10.00 a.m. by ether. Blood was withdrawn from abdominal aorta. Plasma was separated by centrifugation at 3,000 rpm for 10 min at 4°C and used for further analyses of cholesterol, triglyceride and separation of lipoprotein fractions. Liver was removed immediately for preparation of liver microsome for determinations of the cholesterol 7α-hydroxylase activity and for preparation of lipid extract for analyses of liver cholesterol and triglyceride contents. Rectum was also removed to collect feces for analyses of cholesterol and bile acid contents

2.6 Preparation of liver homogenate and liver microsome

Liver homogenate was prepared for determination of free cholesterol, cholesterol ester and triglyceride contents. Liver microsome was prepared from the homogenate for

assay of cholesterol 7α-hydroxylase activity. Since the enzyme activity is subjected to diurnal variation (82), the time of sacrifice was set between 08.00-10.00 a.m.

Preparation of liver microsome was modified from the method of Hoeg *et al* (83). After a hamster was sacrificed, the liver was rapidly excised, and placed in a cooled beaker and all processes were carried out at 4°C. The liver was washed with an ice-cold 0.95% NaCl, blotted dry and weighed. Thereafter, it was finely cut and homogenized with 4 volumes of ice-cold homogenized medium containing 0.25 M sucrose, 1 mM EDTA and 10 mM Tris-HCl pH 8.0 using an ice-chilled Potter Elvehjem glass homogenizer with seven strokes of motor driven Teflon pestle. An aliquot of liver homogenate (1 volume) was taken for lipid extraction and 3 volumes was centrifuged for 50 min at 12,000 x g (9,500 rpm, SS-34 rotor in Iran Sorvall, Inc., California, USA.) and supernatant was filtered through glass wool. The filtrate was further centrifuged at 100,000 x g (40,000 rpm) for 60 min in Beckman 18-55 ultracentrifuge (Beckman Instrument, Inc, Polo Alto, California, USA.) using Ti 60 rotor. The resulting supernatant was discarded. The microsomal pellet was resuspended and homogenized in resuspending buffer (1 ml/g of liver wt) containing 150 mM NaCl, 10 mM Tris-HCl pH 8.0. Liver microsome suspension was determined for protein concentration by the modified method of Lowry (6), and was adjusted to the protein concentration of approximately 20 mg/ml. Small aliquots of resuspended microsomes with known protein concentration were kept at -70°C until used.

2.7 Determination of protein

Total protein of liver microsome was determined by the modified procedure of Lowry (85) using bovine serum albumin as standard.

For liver microsome, 0.1 ml of diluted sample (1:10 dilution of liver microsome suspension) was diluted to 1.0 ml with distilled water. Then 3 ml of alkaline copper solution containing sodium dodecyl sulfate (SDS) freshly prepared by mixing 100 ml of solution A (2% Na_2CO_3, 0.4% NaOH, 0.16% sodium tartrate and 1% SDS) with 1 ml of 4% $CuSO_4 \cdot 5H_2O$ was added to the diluted protein sample. The solution was mixed and left at room temperature for 10 min. Thereafter, 0.3 ml of diluted Folin-Ciocalteus reagent (1:2 dilution with water) was added to the 4 ml of copper-treated protein. The content of each tube was vigorously mixed immediately after addition.The tube was incubated for 45 min at room temperature. The blue color obtained was read absorbance at 660 nm against a reagent blank using spectophotometer (Shimadzu UV-160, Tokyo, Japan). The relationship between protein concentration and absorbance (standard curve) is generated by using 0 (reagent blank), 0.2, 0.4, 0.6, 0.8, and 1.0 ml of standard bovine serum albumin (0.1 mg/ml) diluted to final volume of 1.0 ml.

For liver microsome 0.1, 0.2 and 0.3 ml of diluted microsome suspension (1:100 dilution) was employed in the assay of protein concentration.

2.8 Lipid extraction

Total lipids were extracted from liver homogenate and liver microsome by the modified method of Bligh and Dyer (84).

Liver homogenate or liver microsome suspension (0.8 ml) was mixed with 2 ml methanol and 1 ml chloroform. The mixture was mixed on Vortex mixer for 30 min at

room temperature to extract lipids from the tissue. The lipid present in the extract was partitioned into a chloroform layer by adding 1 ml each of chloroform and 0.95% NaCl. The suspension was mixed gently by inversion. After centrifugation to allow phase separation, the lower chloroform layer containing total lipids was removed and the upper methanol-water layer was washed twice with 2 ml of chloroform. The chloroform extract was combined and 2 ml of benzene was added in order to covaporized with water residue present in the extract. After mixing, the cloudy solution was evaporated to dryness under N_2 at 40-45^{O}C. The lipid residue was weighed and dissolved in chloroform-methanol (9:1, v/v) at concentration of 1-5 mg/ml.

The lipid solution obtained must be clear and contain no protein precipitate. If not, equal volume of benzene was added and the mixture was centrifuged. The clear supernate was removed and evaporated to dryness under N_2 at 40-45°C. The lipid residue was weighed and dissolved in chloroform-methanol (9:1 v/v) at the above concentration. The total lipid extract was kept at -20°C.

2.9 Determination of plasma cholesterol and triglyceride

2.9.1 Determination of plasma cholesterol

Cholesterol in plasma was determined by enzymatic method as described by Allain *et al.* (87) using commercial diagnostic kit (Reagent kit, BM-Lab Ltd., Bangkok, Thailand). Ten microliters each of plasma and various concentrations of standard cholesterol (100, 200, and 300 mg/dl in glacial acetic acid) were mixed with 1.5 ml of the "enzyme mixture" consisting of 0.15 units/ml cholesterol esterase, 0.2 units/ml cholesterol oxidase, 5 units/ml peroxidase, 0.01% 4-aminoantipyrine, 0.05% phenol

and 0.1% Triton X-100 in 0.1 M phosphate buffer pH 7.0. The mixtures were incubated at 37°C for 10 min, thereafter the pink solution obtained was measured absorbance at 550 nm against a reagent blank by using spectrophotometer (Shimadzu UV-160, Tokyo, Japan) (Appendix I).

2.9.2 Determination of plasma triglyceride

Triglyceride concentration in plasma was determined by enzymatic method as described by McGowan *et al.* (90), using commercial diagnostic kit (Reagent kit, BM- Lab Ltd., Bangkok, Thailand.) (Appendix II). Ten microliters each of plasma and various concentrations of standard triglyceride (150, 300 and 450 mg/dl) were mixed with 1.5 ml of the "enzyme mixture". The enzyme mixture was prepared in Tris HCl buffer (50 mmol/L, pH 7.6) containing per liter of 0.1 g. TritonX-100; 1 mmol of 4 amino-antipyrine; α-cyclodextrin; 1.5 mmol sodium 2-hydroxy-3, 5-dichlorobenzene-sulfonate; 5 mmol $MgCl_2$; 0.5 mmol ATP; 10^4 units peroxidase; $0.25x10^3$ units glycerol kinase and 10^5 units of lipase. The mixtures were incubated at 37°C for 5 min and measured absorbance at 520 nm against a reagent blank using spectrophotometer (Shimadzu UV-160, Tokyo, Japan).

2.10 Determination of total and free cholesterol in liver

Total cholesterol content of liver homogenate, liver microsome and free cholesterol content of liver homogenate were determined from its lipid extract by the enzymatic method (86).

Total cholesterol was determined by using an aliquot of total lipid extract in $CHCl_3/CH_3OH$ solution (0.1-0.3 mg lipid) which was blown down to dryness under N_2.

Thereafter, 10 µl of 5% Triton X-100 and 10 µl of isopropanol was added into sample and blank tube to dissolve lipid residue, then 1.5 ml of the enzyme mixture (Appendix I) was added. For standard tube, 10 µl of standard cholesterol 100, 200 and 300 mg/dl in glacial acetic acid and 10 µl of isopropanol were added to the enzyme mixture and the reaction was performed in the same way as the samples.

Free cholesterol content was determined in the same way as total cholesterol except cholesterol esterase was omitted from the enzyme mixture.

2.11 Assay of cholesterol 7α-hydroxylase activity

Cholesterol 7α-hydroxylase activity was measured by the modified method of Princen *et al.* (88).

300 µl of (4-^{14}C)-Cholesterol (specific activity 1.54 x 10^3 dpm/nmole) containing 344 nmole cholesterol (purified prior to use) was pipetted into a 15 ml reaction tube. The solvent was evaporated to dryness under N_2. The ^{14}C-cholesterol residue was carefully suspended in 200 µl of Tween 80 (7.5 mg/ml) by constant shaking (150 rpm) for 60 min at 40°C. The suspension obtained was preincubated with 0.1 ml of liver microsome (≈ 2 mg protein), 200 µl of buffer (containing 100 mM sucrose, 40 mM potassium phosphate, pH 7.2, 30 mM EDTA, 20 mM dithiothereitol and 50 mM KF) and 50 µl of distilled water at 37°C with constant shaking (150 rpm) for 15 min to allow complete mixing of (4-^{14}C)-cholesterol with the endogenous cholesterol pool. Assay was then started by adding NADPH-generating system containing 20 mM glucose-6-phosphate dehydrogenase, 2 mM $NADP^+$, 4 mM $MgCl_2$, 1.4 IU glucose-6-phosphate dehydrogenase and 5 M potassium phosphate buffer pH 7.4 in a final volume

of 1.0 ml. The incubation was carried out at 37°C with constant shaking (150 rpm) for 30 min. The reaction was stopped by adding 2 ml 95% ethanol and the mixture was mixed vigorously for 2 min. The ^{14}C-cholesterol substrate and oxidative products were extracted by 2 ml petroleum ether. The upper petroleum ether layer containing cholesterol and oxidative products was removed and the lower layer was washed twice with 2 ml petroleum ether. The combined petroleum ether extract was evaporated to dryness under N_2.

The dry residue obtained was redissolved in a small volume of chloroform (0.2 ml). At this state, 90-95% of the radioactivity was recovered from the incubation mixture. The petroleum ether extract was chromatographed on silica gel G thin-layer plate (0.5 mm thickness) along with 40 µl of 20 mg/ml cholesterol standard. The TLC plate was twice developed in solvent system of benzene/ethylacetate (2:3 v/v). The lipid spots were visualized by exposing the dried plate with iodine vapor in a closed container. The silica gel in the band corresponding to 7α-hydroxycholesterol (Rf ≈ 0.65) was scraped out and extracted 3 time with diethyl ether. Combined extract was dried and extracted 3 time with diethyl ether. Combined extract was dried and transferred to a glass counting vial containing 5.0 ml of scintillation fluid (0.35% PPO, 0.05% POPOP) in toluene for radioactivity counting. Each remaining band was also scraped out and counted for radioactivity. The radioactivities were measured by using Beckman LS 1801 Liquid Scintillation counter (Beckman instruments, Inc, Palo Alto, California, USA.). The control tube containing boiled microsome was performed simultaneously to correct for the non-enzymatic oxidation of (4-^{14}C)-cholesterol substrate.

Cholesterol 7α-hydroxylase activity was expressed as % conversion of radioactivity from (4-^{14}C)-cholesterol to 7α-hydroxycholesterol under the assay condition. The % conversion in the assay and control tubes were calculated separately from

$$\frac{\text{dpm in } 7\alpha\text{ - hydroxycholesterol}}{\text{total dpm recovered from the TLC plate}} x100 = \%\text{conversion}$$

The cholesterol 7α-hydroxylase activity = % conversion in the assay tube - % conversion in control tube

2.12 Determination of biliary lipid in feces

2.12.1 Collection of feces

At the time of sacrifice, feces were collected individually from cecum. It was dried overnight at 50°C and dehydrated by soda lime in a desicator until a stable weight of feces was obtained. Fecal sample was kept for further analyses of cholesterol and bile acid content.

2.12.2 Determination of fecal cholesterol content

Cholesterol in feces was determined by enzymatic method (87). Fecal sample (100-150 mg.) was homogenized in 1 ml of 0.067 M phosphate buffer pH 7.0. Then 1 ml of extraction solvent consisting of ethyl acetate and ethanol (1:1 v/v) was added. The sample was extracted twice and centrifuged at 2,500 rpm for 5 min. The supernatant was transferred into a tube and the lower layer was washed twice with 1 ml of the extract solvent mixture. The supernatant was combined and assayed for cholesterol concentration by using enzymatic method as previously described (87).

2.12.3 Determination of fecal bile acid content

Bile acids were extracted from feces and analyzed by the modified method of Turnberg and Mote, 1969 (89).

The feces was ground in a mortar and suspended in 20 ml 0.067 M phosphate buffer pH 7.0 (feces 20 mg: 2 ml of buffer). Then 5 ml of methanol was added, mixed and warm in a water-bath for 5 min at 37°C. The suspension was centrifuged at 3,000 rpm for 5 min. The supernatant was collected and precipitate was washed twice with 2 ml methanol. The combined methanolic extract was evaporated under N_2 and residue was extracted three times with petroleum ether to remove non-polar compounds. The petroleum ether extract was discarded. The residue containing bile acids was added 1.5 ml of 50% ethanol and kept for further analysis of bile acid content (Appendix III).

2.13 Separation of plasma lipoprotein fractions

Plasma lipoproteins were separated by sequential ultracentrifugation technique. This method was modified by using the formula of Havel *et al*, (80) in calculating density. Only 0.5 ml of plasma was separated into fractions of very low density (VLDL, d<1.019 g/ml), low density (LDL, d=1.019-1.063 g/ml), and high density lipoproteins (HDL, d>1.063 g/ml) by TLA-100.2 fix angle rotor in TL-100 Ultracentrifuge. These fractions were completely performed within 14 hrs at 4°C.

The plasma was adjusted to the desired density by the addition of concentrated salt solution. Two stock salt solutions were prepared as, described below.

A. Mock solution, a solution with the same background salt density as plasma (d=1.0063 g/ml) contained 1.142 g of NaCl and 10 mg of Na_3EDTA in 100 ml distilled water.

B. Concentrated stock salt solution (d=1.346 g/ml) contained 35.4 g of potassium bromide, 15.3 g sodium chloride and 10 mg of Na_3EDTA in 100 ml distilled water.

From both solutions, the required salt solution was prepared according to the following formula.

$$A \cdot Y + B \cdot Z = (A+B)X$$

Where X was the desired density of mixture, A and B were the volume of the stock solution A and B, Y and Z were their densities, respectively. Serial preparation of individual lipoprotein fractions were run by ultracentrifugation in TLA-100.2 fix angle rotor in TL-100 ultracentrifuge (Beckman instrument, Inc., Polo Alto, California, USA.) as shown in the protocol below (Figure N) (81). The plasma was adjusted to required density with salt solution and transferred to 1 ml open top thick wall polycarbonate tube. After balancing, the loaded rotor was placed into the TL-100 and the run parameter was set according to the protocol in Figure N. Upon completion of the run, each tube was carefully removed from the rotor so as not to disturb the layers. Then 0.5 ml of lipoprotein fraction in the top layer was separated from the bottom using capillary pipette. The separated fraction was mixed and kept in freezer at -20°C for further determination of cholesterol concentration.

Separation of plasma lipoproteins by TL-100 ultracentrifuge

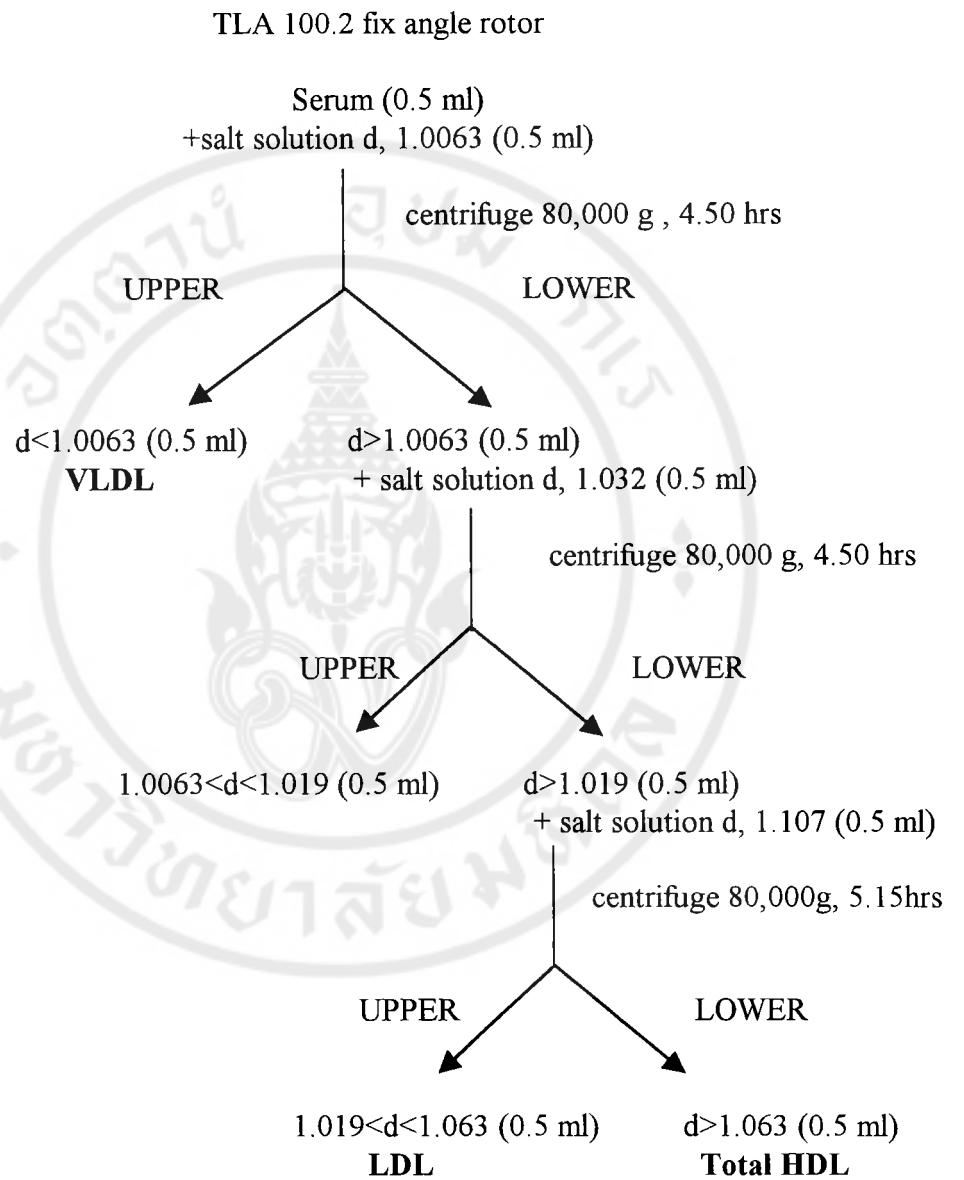


Figure N: Flow chart for the separation of lipoproteins using ultracentrifuge.

2.14 Statisticals

All data were expressed as mean±standard error of mean ($\bar{X}$±SEM). The significant difference between groups was analyzed by using the one way analysis of variance (one way ANOVA). Unpaired student's t-test was used for comparison between two groups of means in control and experimental groups. Whereas the difference within a group was evaluated by using student's paired t-test. Difference was considered to be statistically significant if the probability value was less than 0.05 ($p<0.05$) and was highly significant when the P-value was less than 0.01 ($p<0.01$). All tests were conducted by using SPSS program for Window.

CHAPTER III

RESULT

1. Induction of hypercholesterolemia in hamsters by cholesterol supplementation

This experiment was designed to induce hypercholesterolemia in hamsters for testing the hypocholesterolemic effect of, 2,6-DHA on plasma cholesterol. In this experiment, adult male hamsters, body weight in the range of 80-120 g were divided into two groups, namely hypercholesterolemic and control groups. In hyper-cholesterolemic group, the animals were orally supplemented with cholesterol dissolved in corn oil at 0.2% body weight everyday for 3 weeks, whereas in the control group, animals were supplemented with equal amount of corn oils. Both groups were fed with the same pelleted diet. Body weight and plasma cholesterol concentration of two groups were monitored every week during the experimental period.

Figure 1 shows body weight of hamsters orally supplemented with either cholesterol (0.2% BW) in corn oil (chol-fed) or with only corn oil supplementation (control). As shown in Figure 1, body weight of hamsters in chol-fed group and control group were slightly increased approximately 10% in 3 weeks. There were no significant difference in body weight between both groups of animals and they were healthy and showed no sign of sickness.

Figure 2 shows total plasma cholesterol concentration (mg/dl) of hamsters orally supplemented with cholesterol (0.2% BW) in corn oil (chol-fed) and with only corn oil

Table 1. Body weight of hamsters supplemented with cholesterol in corn oil (0.2% BW) and only corn oil supplemented on everyday.

Body weight (g)								
	Control (corn oil-fed)				Cholesterol-fed (0.2% BW)			
No	0 wk	1 wk	2 wk	3 wk	0 wk	1 wk	2 wk	3 wk
1	97.0	100.4	102.7	104.6	97.1	97.6	98.7	103.1
2	96.0	98.2	101.4	108.2	96.6	99.5	105.1	112.0
3	96.3	103.2	108.4	111.0	96.1	102.1	107.4	110.7
4	91.1	93.0	97.0	101.0	90.0	92.9	94.8	95.6
5	90.0	95.2	98.2	100.2	90.0	90.8	93.6	100.3
6	91.6	94.7	95.0	96.4	90.3	90.5	90.6	91.7
7	103.5	104.8	106.4	110.0	103.9	105.7	107.3	110.4
8	101.0	104.0	106.4	109.3	101.8	103.7	110.4	113.5
9	98.0	99.3	103.2	105.4	100.6	104.2	107.7	108.9
10	92.6	95.4	100.3	102.4	94.6	100.6	102.3	105.6
Mean $\pm$ SEM	95.7 $\pm$1.4	98.8 $\pm$1.3	101.9 $\pm$1.4	104.9 $\pm$1.5	96.1 $\pm$1.6	98.8 $\pm$1.8	101.8 $\pm$2.2	105.2 $\pm$2.3

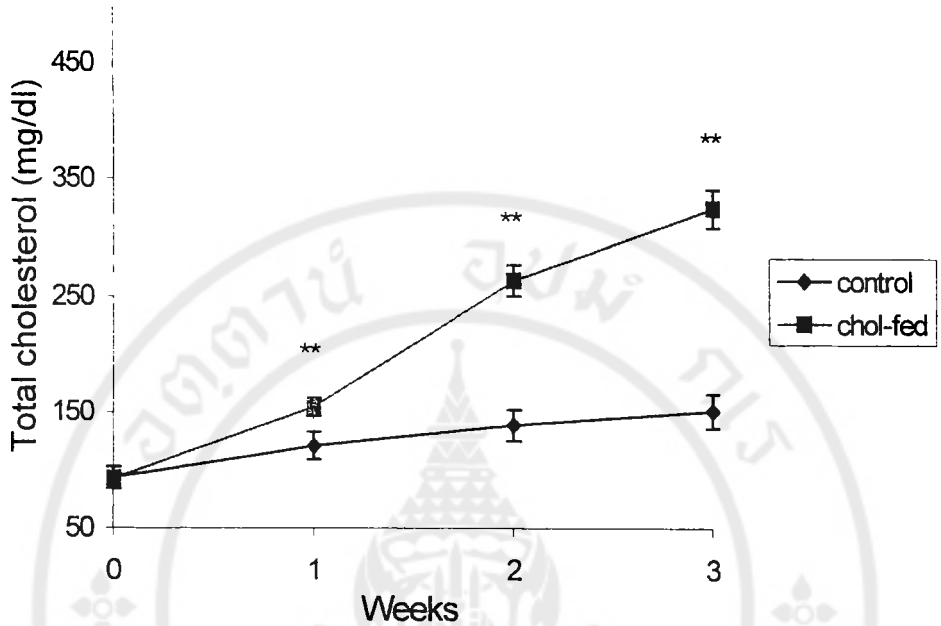


Figure 2. Total plasma cholesterol concentration (mg/dl) of hamsters orally supplemented with cholesterol 0.2% BW in corn oil (chol-fed) and with only corn oil (control).

Value are means ± SEM obtained from 10 animals.

**P<0.001 significant difference from the control group at corresponding time.

Table 2. Total plasma cholesterol concentration (mg/dl) of hamsters supplemented with cholesterol in corn oil (0.2% BW) and only corn oil supplemented on everyday.

Plasma cholesterol (mg/dl)								
	Control (corn oil-fed)				Cholesterol-fed (0.2% BW)			
No		1 wk	2 wk	3 wk	0 wk	1 wk	2 wk	3 wk
1	80.1	124.2	129.4	140.0	81.4	135.2	265.2	305.4
2	108.0	131.2	143.0	182.0	102.2	167.3	270.4	333.2
3	72.3	99.4	139.0	140.2	77.2	159.3	226.2	325.1
4	87.2	101.2	131.0	140.3	88.0	131.1	265.2	326.1
5	102.0	116.1	129.0	132.2	102.0	165.2	298.0	347.2
6	88.0	100.1	129.0	156.2	84.2	197.0	265.0	302.2
7	81.2	136.0	139.4	150.2	82.2	156.2	259.0	320.0
8	90.4	112.3	124.5	143.4	90.1	143.0	259.1	320.3
9	110.0	135.0	157.1	160.0	111.2	140.4	263.2	343.1
10	108.0	148.0	163.1	164.3	100.7	151.2	261.4	321.0
Mean ± SEM	92.7 ± 4.2	120.4 ± 5.4	138.4±4.0	150.9±4.7	91.9 ±3.6	154.6**±6.1	263.3**±5.5	324.4**±4.5

** P<0.001 significant difference from the normal control group at corresponding time.

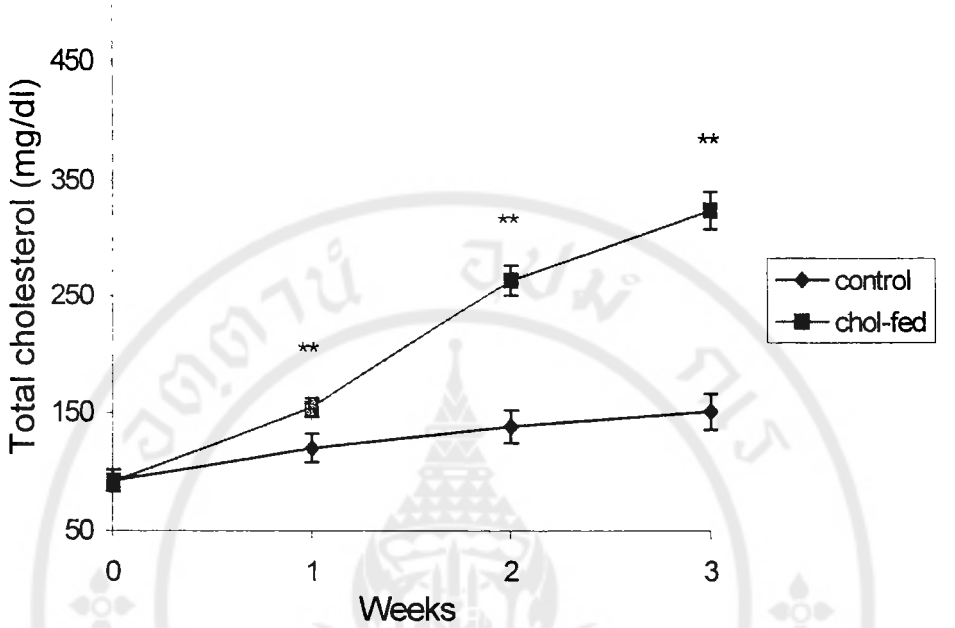


Figure 2. Total plasma cholesterol concentration (mg/dl) of hamsters orally supplemented with cholesterol 0.2% BW in corn oil (chol-fed) and with only corn oil (control).

Value are means ± SEM obtained from 10 animals.

**P<0.001 significant difference from the control group at corresponding time.

(control). During 3 weeks of feeding period, total plasma cholesterol concentration in cholesterol supplemented group were constantly increased to the level 324.4±4.5 mg/dl at the end of 3 weeks, whereas the plasma cholesterol concentration of control group was relatively unchanged. The total plasma cholesterol concentration of chol-fed group were highly significant difference ($p<0.001$) from the non-supplement group after one week of cholesterol supplementation. The individual body weight and plasma cholesterol of hamsters in both groups were shown in Tables 1 and 2 respectively.

The result in this experiment indicated that adult male hamsters could be induced to be hypercholesterolemia within 3 weeks by orally supplemented with cholesterol (0.2% BW) in corn oil. For this period, their plasma cholesterol concentration were elevated to level approximately 300 mg/dl without any sign of sickness.

2. Dose response effect of 2,6-DHA on lowering plasma cholesterol and triglyceride in hypercholesterolemic hamsters

This experiment was designed to determine the effective dose of 2,6-DHA on lowering plasma cholesterol and triglyceride concentrations in hypercholesterolemic hamsters. In this experiment, hypercholesterolemic hamsters having plasma cholesterol and triglyceride concentrations in the range of 250-350 mg/dl and 400-500 mg/dl respectively were divided into two groups namely treated and non-treated with 2,6-DHA. In the treated group, animals were orally administered with various doses of 2,6-DHA at 300, 400, 600 and 800 μmol/kg BW. The test compound was dissolved in 10% ethanol and 90% corn oil and was given twice a day (10.00 a.m. and 3.00 p.m.)

for 7 days whereas in the non-treated group, animals were orally administered with equal amount of placebo (10% ethanol and 90% corn oil). Both groups of hypercholesterolemic hamsters were continuously maintained on cholesterol (0.2% BW) supplement. In addition, normal control hamsters which received only the placebo but no cholesterol (0.2% BW) supplement were also maintained at the same time to see the effect of placebo on plasma cholesterol and triglyceride.

Table 3 shows the effect of 2,6-DHA on body weight, liver weight, plasma cholesterol and triglyceride concentration. Body weight of every group of animals, normal control, non-treated (hypercholesterolemic control) and treated at various doses showed no significant difference between groups except for those receiving high dose (800 μmol/kg BW) which was lower than the corresponding control group approximately 15% after 7 days of treatment. When liver weight were compared, there was no significant difference among animals received only normal diet, cholesterol supplement with and without 2,6-DHA administration.

In the normal control hamsters, plasma cholesterol and triglyceride were approximately 150 and 180 mg/dl respectively. After receiving placebo for 7 days, there was no change in plasma cholesterol level but plasma triglyceride was slightly increased. In the cholesterol-fed group, hamsters were maintained on cholesterol supplement and received no 2,6-DHA but only placebo for 7 days, plasma cholesterol and triglyceride were increased to approximately 370 and 860 mg/dl respectively. Upon receiving 2,6-DHA at various doses (300-800 μmol/kg BW), the plasma cholesterol and triglyceride decreased in a dose dependent manner.

Table 4 and Figure 3 show dose response effect of 2,6-DHA on lowering plasma cholesterol in hypercholesterolemic hamsters after 7 days of treatment. The data

Table 3. Effect of 2,6 DHA supplemented at various doses for 7 days on body weight, liver weight, plasma cholesterol and plasma triglyceride of hypercholesterolemic hamsters.

Parameter	Normal control		Cholesterol-fed				
	Control A (corn oil)	Control B (corn oil+solvent)	2,6-dihydroxyacetophenone (μmol/kg BW)				
			0	300	400	600	800
Body weight (g)	87.8 ± 2.6	86.9 ± 3.6	87.0 ± 5.0	87.7 ± 3.7	84.7 ± 5.2	85.4 ± 2.8	72.8 ± 2.4
Liver weight (g/100 g B W)	3.3 ± 0.1	3.3 ± 0.1	3.7 ± 0.2	3.7 ± 0.2	3.6 ± 0.1	3.6 ± 0.2	3.4 ± 0.1
Plasma cholesterol (mg/dl)	150.9 ± 4.7	149.4 ± 47.7	372.4 ± 22.6*	261.8 ± 15.5^{++}	209.3 ± 14.8**$^{++}$	198.9 ± 15.3**$^{++}$	198.5 ± 18.8**$^{++}$
Plasma triglyceride (mg/dl)	181.0 ± 6.6	187.3 ± 17.1	865.9 ± 158.2*	367.1 ± 36.2*$^{++}$	279.7 ± 30.2**$^{++}$	264.1 ± 33.0**$^{++}$	282.2 ± 28.1**$^{++}$

Values are means ± SEM obtained from 10 animals.

* $P<0.05$, ** $P<0.001$ significant difference from the value before treatment

$^{++}$ $P<0.001$ significant difference from hypercholesterolemic (non- treated) control.

Control A, B: Animals were maintained on normal diet without cholesterol supplement and were given corn oil and corn oil + solvent for DHA, respectively.

showed that plasma cholesterol concentration in non-treated group (control) was increased from 284.8±2.2 mg/dl to 372.4±27.6 (≈30%) after treatment with placebo whereas in the treated group which received 2,6-DHA at doses of 300, 400, 600 and 800 μmol/kg BW showed dose dependent effect on lowering plasma cholesterol when the dose of 2,6-DHA were in the range of 300-600 μmol/kg BW. The hypo-cholesterolemic effect of 2,6-DHA was maximum at dose 600 μmol/kg BW because at higher dose of 800 μmol/kg BW, there was no change in its ability to decrease plasma cholesterol as compared to 600 μmol/kg BW dose. The cholesterol lowering effect of 2,6-DHA was not significantly different with the dose 300 μmol/kg BW whereas there were highly significant difference ($p<0.001$) at doses 400, 600 and 800 μmol/kg BW before and after treatment. However treatment with 2,6-DHA at all doses (300-800 μ mol/kg BW) showed ability to lowering plasma cholesterol when compared with the non-treated group (control). In comparison with the non-treated group, treatment of 2,6-DHA at 300, 400, 600 and 800 μmol/kg BW were able to decrease plasma cholesterol to 71.0%, 53.6%, 50.0% and 52.7% respectively within 7 days (Table 4).

For plasma triglyceride, Table 5 and Figure 4 also showed dose response effect of 2,6-DHA on lowering plasma triglyceride in hypercholesterolemic hamsters after 7 days of treatment. In non-treated group (control), plasma triglyceride was significantly increased ($p<0.05$) from 435.3±39.4 mg/dl at the beginning to 865.9±158.2 mg/dl (≈ 100% increased) after receiving normal diet supplemented with cholesterol in corn oil (0.2% BW) for 7 days. In contrast, the group received 2,6-DHA at various doses for 7 days, plasma triglyceride was decreased in a dose dependent manner. The observed plasma triglyceride level of 2,6-DHA treated at 300, 400, 600 and 800 μmol/kg BW were

Table 4. Effect of 2,6-DHA supplemented at various doses for 7 days on total plasma cholesterol level of hypercholesterolemic hamsters.

2,6-DHA (μmol/kg BW)	Plasma cholesterol (mg/dl)		% cholesterol after treatment	% of control
	Before	After		
0 (control)	284.8 ± 2.2	372.4 ± 27.6*	130.6 ± 7.4*	100
300	284.3 ± 8.9	261.8 ± 15.5^{++}	92.7 ± 6.0^{++}	71.0
400	297.4 ± 6.7	209.3 ± 14.8**$^{++}$	70.0 ± 4.0**$^{++}$	53.6
600	305.4 ± 7.1	198.9 ± 15.3**$^{++}$	64.9 ± 4.4**$^{++}$	50.0
800	286.4 ± 6.4	198.5 ± 18.8**$^{++}$	68.8 ± 5.3**$^{++}$	52.7

Values are means ± SEM obtained from 10 animals.

* P<0.05, ** P<0.001 significant difference from the value before treatment at corresponding dose.

$^{++}$ P<0.001 significant difference from the non-supplemented (control) group.

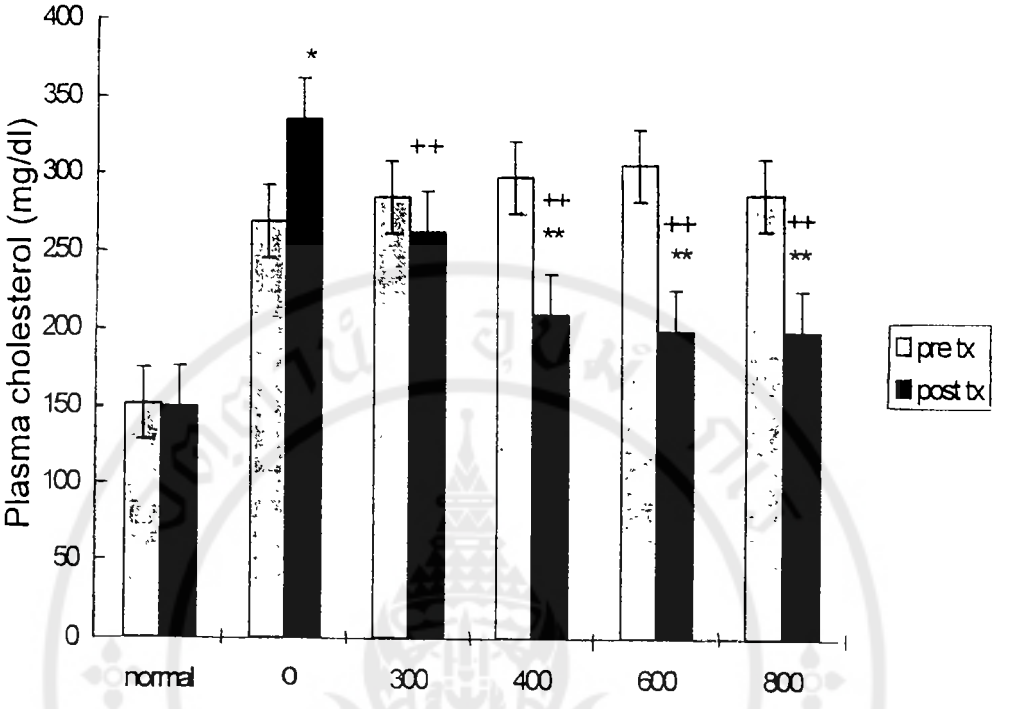


Figure 3. Dose response effect of 2,6-DHA : 300, 400, 600 and 800 μmol/kg BW supplemented for 7 days on plasma cholesterol concentration (mg/dl) of hypercholesterolemic hamsters.

Values are mean ± SEM obtained from 10 animals.

*P<0.05, **P<0.001 significant difference from the value before treatment.

++P<0.001 significant difference from hypercholesterolemic (non-treated) control.

In normal group, animals were maintained on normal diet without cholesterol supplement but were given corn oil (pre treatment) and corn oil+solvent for 2,6-DHA (post treatment) for 7 days.

Table 5. Effect of 2,6 DHA supplemented at various doses for 7 days on total plasma triglyceride level of hypercholesterolemic hamsters.

2,6 DHA (μmol/kg BW)	Plasma triglyceride (mg/dl)		% triglyceride after treatment	% of control
	Before	After		
0 (Control)	435.3±39.4	865.9±158.2*	213.0±48.8*	100
300	489.5±27.0	367.10±36.2*$^{++}$	75.8±7.1*$^{++}$	35.6
400	426.9±38.6	256.8±26.9**$^{++}$	58.3±6.2**$^{++}$	27.4
600	475.42±31.8	264.1±33.0**$^{++}$	55.1±5.3**$^{++}$	25.9
800	472.4±27.3	282.2±28.1**$^{++}$	61.4±6.2**$^{++}$	28.8

Values are means ± SEM obtained from 10 animals.

* $P<0.05$, ** $P<0.001$ significant difference from the value before treatment at corresponding dose.

$^{++}$ $P<0.001$ significant difference from the non-supplemented (control) group.

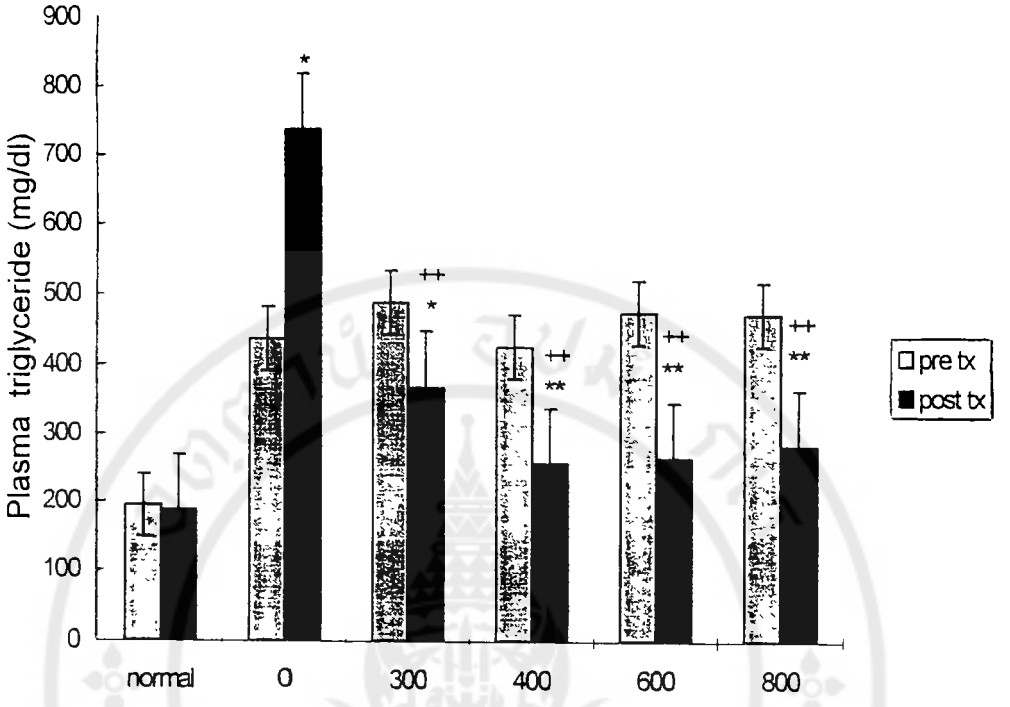


Figure 4. Dose response effect of 2,6-DHA : 300, 400, 600 and 800 μmol/kg BW supplemented for 7 days on plasma triglyceride concentration (mg/dl) of hypercholesterolemic hamsters.

Values are mean ± SEM obtained from 10 animals.

*P<0.05, **P<0.001 significant difference from the value before treatment.

++P<0.001 significant difference from hypercholesterolemic (non-treated) control.

In normal group, animals were maintained on normal diet without cholesterol supplement but were given corn oil (pre treatment) and corn oil+solvent for 2,6-DHA (post treatment) for 7 days.

decreased from 489.5 ± 27.0 mg/dl, 426.9 ± 38.6 mg/dl, 475.42 ± 31.8 mg/dl and 472.4 ± 27.3 mg/dl before treatment to 367.1 ± 36.2 mg/dl, 256.8 ± 26.9 mg/dl, 264.1 ± 33.0 mg/dl and 282.2 ± 28.1 mg/dl after treatment, respectively. The ability to reduce plasma triglyceride of 2,6-DHA was maximum at 600 μmol/kg BW because as the dose was increased to 800 μmol/kg BW, the plasma triglyceride was not decreased any more but rather increased. The level of plasma triglyceride in every group of animals after 2,6-DHA treated (300-800 μmol/kg BW) showed highly significant difference from those obtained before treatment and showed highly significant difference ($p<0.001$) from the non-treated group (control). The plasma triglyceride of 2,6-DHA treated at doses 300, 400, 600 and 800 μmol/kg BW decrease to 35.6 %, 27.4%, 25.9% and 28.8% of the corresponding controls, respectively when compared with that of non-treated group.

The result in this experiment demonstrated that 2,6-DHA exhibited a dose dependent effect on lowering plasma cholesterol and triglyceride levels. The minimum effective dose of 2,6-DHA on reducing plasma cholesterol and triglyceride levels was 400 μmol/kg BW that could reduce both plasma cholesterol and triglyceride by approximately 46% and 73% respectively.

3. Time course effect of 2,6-DHA on plasma lipids in hyper-cholesterolemic hamsters

This experiment was designed to determine the time course effect of 2,6-DHA on lowering plasma cholesterol and triglyceride in hypercholesterolemic hamsters. The animals were treated with 2,6-DHA at dose 400 μmol/kg BW which was the minimum effective dose on lowering plasma cholesterol and triglyceride after 7 days of treatment.

In this experiment hypercholesterolmic hamsters which had plasma cholesterol in the range of 250-350 mg/dl and plasma triglyceride 400-500 mg/dl were divided into two groups, treated and non-treated group (control). In the treated group, animals were supplemented with 2,6-DHA at 400 μmol/kg BW in 10% ethanol and 90% corn oil for 14 days whereas in the control group animals were given only placebo (10% ethanol and 90% corn oil) in the same manner. Both groups were maintained with cholesterol (0.2% BW) supplement during the course of treatment. Body weight, plasma cholesterol and triglyceride were measured before treatment (day 0) and during the course of treatment at day 3, 7, 10 and 14.

Figure 5 shows body weight of hypercholesterolemic hamster treated with 2,6-DHA at dose 400 μmol/kg BW and non-treated (control) for 14 days. The result showed that body weight of two groups at the beginning were not significant difference, which was approximately 107 ± 2.3g and 103 ± 1.1g in non-treated and treated groups, respectively. During 14 days of treatment, body weight of both groups slightly declined to approximately 95 ± 2.6 g in non-treated group and 93 ± 0.8 g in treated group but there was no significant difference between both groups.

Table 6 and Figure 6 show time course effect of 2,6-DHA on plasma cholesterol. In control group, hypercholesterolemic hamsters maintained on cholesterol supplement with out 2,6-DHA treated, plasma cholesterol were constantly increased at the beginning of placebo administration from 268.6 ± 5.7 mg/dl, to 313.7 ± 6.2 mg/dl, 335.1 ± 7.2 mg/dl, 352.9 ± 14.8 mg/dl and 377.7 ± 11.9 mg/dl on day 3, 7, 10, and 14 respectively whereas supplementation with 2,6-DHA at 400 μmol/kg BW, plasma

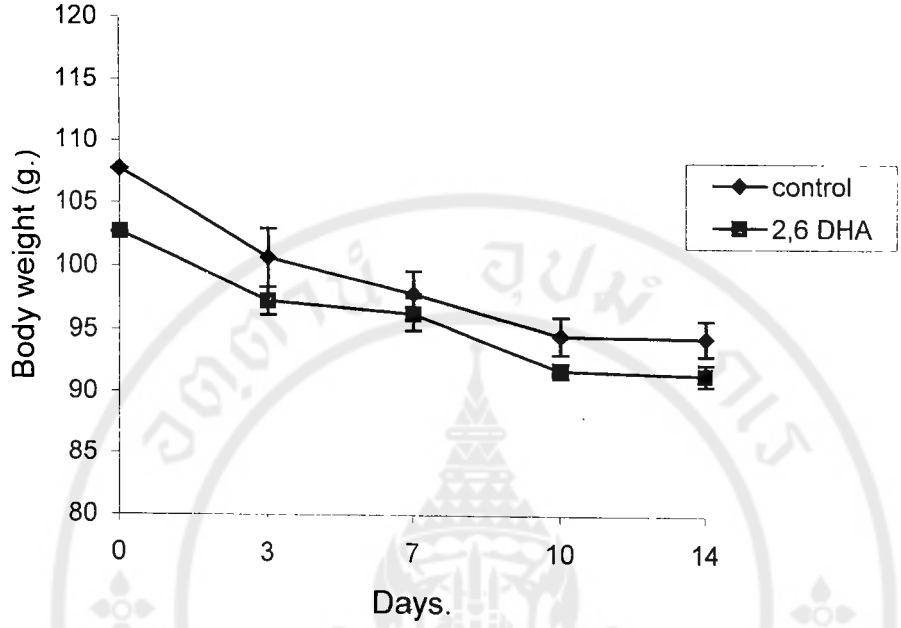


Figure5. Time course effect on body weight of hamsters treated and non-treated (control) with 2,6-DHA 400 μmol/kg BW for 14 days.

cholesterol level was constantly declined after 3 days of treatment and was highly significant difference ($p<0.001$) when compared with the control group. Plasma cholesterol in 2,6-DHA treated was 274.4 ± 5.5 mg/dl on day 0 and decreased to 230.4 ±6.3 mg/dl, 194.7 ± 7.5 mg/dl, 154.3 ± 3.8 mg/dl and 147.5 ± 3.3 mg/dl or 84.0 ± 1.7%, 71.0 ± 2.2%, 57.2 ± 1.1% and 54.1 ± 1.2 % on day 3, 7, 10 and 14 respectively.

For plasma triglyceride level as shown in Table 6 and Figure 7, in control group which was maintained on cholesterol in corn oil supplement, it was constantly increased in the same manner as in the case of plasma cholesterol from 437.7 ± 19.8 mg/dl on day 0 to 632.2 ± 26.9 mg/dl, 738.6 ± 40.0 mg/dl, 744.3 ± 39.5 mg/dl and 849.9 ± 55.4 mg/dl on day 3, 7, 10 and 14 respectively whereas in 2,6-DHA treated group, plasma triglyceride was constantly declined and was highly significant difference ($p<0.001$) when compared with the control group. Plasma triglyceride of 2,6-DHA treated was decreased from 436.9 ± 22.3 mg/dl at the beginning (day 0) to 327.4 ± 14.6 mg/dl, 236.7 ± 11.8 mg/dl, 150.4 ± 12.2 mg/dl and 138.2 ± 9.0 mg/dl or 75.6 ± 2.9%, 54.9 ± 3.1%, 34.9 ± 3.1%and 32.3 ± 2.6% on day 3, 7, 10 and 14 respectively. The result from this experiment also indicated that after 10 days of treatment with 400 μmol/kg BW of 2,6 DHA, plasma cholesterol and triglyceride were not significantly different from day 14 probably plasma lipids level were already reduced to normal levels. Therefore, the maximum hypolipidemic effect on plasma cholesterol and triglyceride level of 2,6-DHA at dose 400 μmol/kg BW was obtained after 10 days of treatment.

Table 6. Time course effect of 2,6 -DHA (400 μmol/kg BW) supplemented on total plasma cholesterol and total plasma triglyceride levels of hypercholesterolemic hamsters.

Time (days)	Plasma cholesterol (mg/dl)				Plasma triglyceride (mg/dl)			
	Control		2,6-DHA treated		Control		2,6-DHA treated	
	mg/dl	% of control after treatment	mg/dl	% cholesterol after treatment	mg/dl	% of control after treatment	mg/dl	% triglyceride after treatment
0	268.6 ± 5.7	100.0 ± 0.0	274.4 ± 5.5	100.0 ± 0.0	437.7 ± 19.8	100.0 ± 0.0	436.9 ± 22.3	100.0 ± 0.0
3	313.7 ± 6.2	116.9 ± 1.1	230.4 ± 6.3**	84.0 ± 1.7**	632.2 ± 26.9	142.8 ± 3.8	327.4 ± 14.6**	75.6 ± 2.9**
7	335.1 ± 7.2	124.9 ± 2.1	194.7 ± 7.5**	71.0 ± 2.2**	738.6 ± 40.0	169.3 ± 7.1	236.7 ± 11.8**	54.9 ± 3.1**
10	352.9 ± 14.8	132.1 ± 6.5	154.3 ± 3.8**	57.2 ± 1.1**	744.3 ± 39.5	170.6 ± 6.8	150.4 ± 12.2**	34.9 ± 3.1**
14	377.7 ± 11.9	141.5 ± 6 1	147.5 ± 3.3**	54.1 ± 1.2**	849.9 ± 55.4	194.6 ± 10.3	138.2 ± 9.0**	32.3 ± 2.6**

Values are means ± SEM obtained from 10 animals.

** P<0.001 significant difference from zero time value.

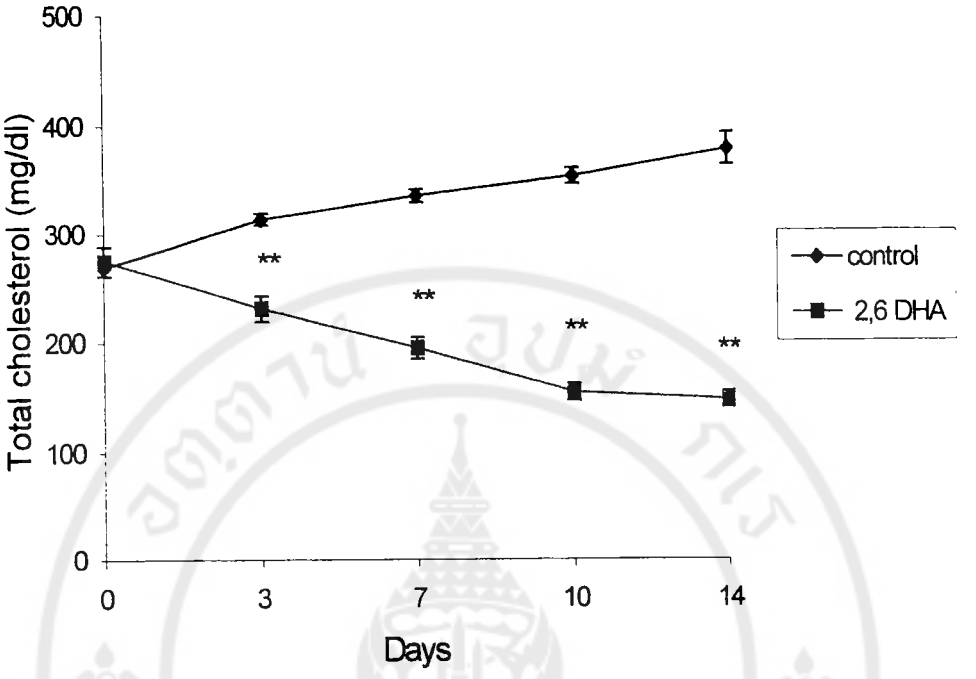


Figure 6. Time course effect on total plasma cholesterol (mg/dl) of hamsters treated and non-treated (control) with 2,6-DHA 400 μmol/kg BW for 14 days.

Values are mean ± SEM obtained from 10 animals

**P<0.001 significant difference from hypercholesterolemic (non-treated) control at corresponding time.

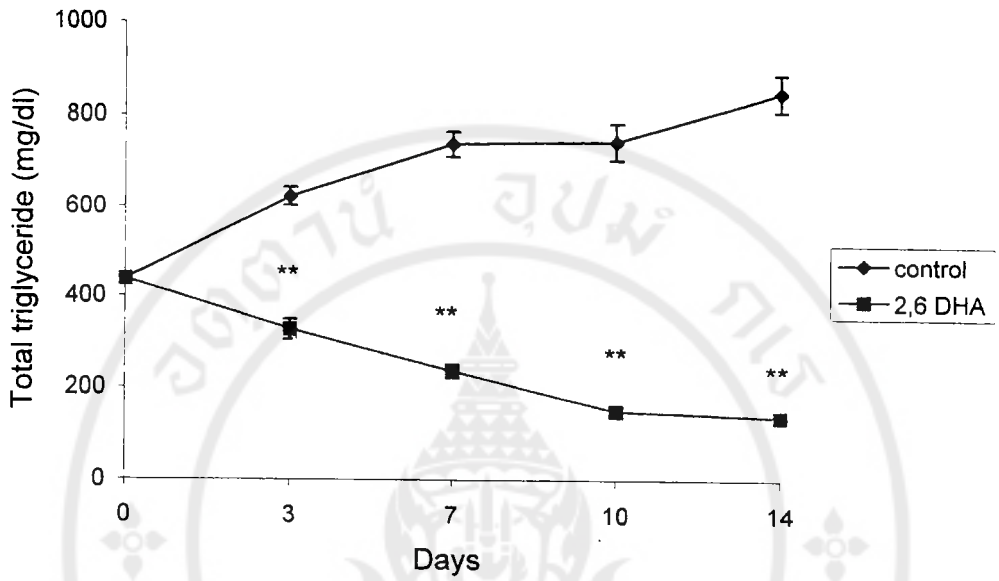


Figure 7. Time course effect on total triglyceride (mg/dl) of hamsters treated and non-treated (control) with 2,6-DHA 400 μmol/kg BW for 14 days.

Values are mean ± SEM obtained from 10 animals

**P<0.001 significant difference from hypercholesterolemic (non-treated) control at corresponding time.

4. Effect of 2,6-DHA on triglyceride and cholesterol contents in liver

The cholesterol content in liver was important for the regulation of cholesterol metabolism, biosynthesis and degradation. In the cholesterol biosynthesis pathway, cholesterol pool in liver affects HMG-CoA reductase activity which was rate limiting step for cholesterol synthesis whereas in the degradation pathway cholesterol pool in liver act as substrate for bile acid synthesis. For triglyceride, accumulation of triglyceride in liver indicates a defect in excretion of triglyceride in the form of VLDL. Therefore this experiment was designed to find out the effect of 2,6-DHA on triglyceride and cholesterol contents in liver of hypercholesterolemic hamster treated with 2,6-DHA at dose 400 μmol/kg BW for 7 days compared with non-treated (hypercholesterolemic control) and normal control. Both free and esterified cholesterol were analyzed for estimation the activity of acyl CoA-cholesterol acyltranferase (ACAT) which catalyzes cholesteryl ester synthesis.

Table 7 shows the effect of 2,6 DHA supplement for 7 days on liver triglyceride and cholesterol contents. In hypercholesterolemic hamsters, 2,6-DHA treated and non-treated, triglyceride and cholesterol contents in liver were significantly higher than that of normal control (no cholesterol supplement). Hypercholesterolemic hamsters treated with 2,6-DHA show no significant difference in triglyceride and total cholesterol contents when compared with non-treated group. The triglyceride contents in liver of normal control was 3.8±0.3 mg/g liver whereas in hypercholesterolemic hamsters was 5.3±0.3 mg/g liver in non-treated (control) and 5.4±0.4 mg/g liver in 2,6-DHA treated group. Total cholesterol content in liver of normal hamster was 2.3±0.2 mg/g liver whereas in hypercholesterolemic hamstes in both 2,6-DHA treated and non treated were similar approximately 8.0±0.2 mg/g liver and 8.0±0.3 mg/g liver respectively.

Table 7. Effect of 2,6-DHA (400 μmol/kg BW) supplemented for 7 days on liver weight, cholesterol and triglyceride contents in liver.

Group	Liver weight (g/100 BW)	Triglyceride (mg/g liver)	Liver cholesterol (mg/g liver)			Free : Esterified
			Total	Free form	Ester form	
Normal control	3.3 ± 0.1	3.8 ± 0.3	2.3 ± 0.2	1.6 ± 0.2	0.7 ± 0.1	2.4 ± 0.2
Hypercholesterolemic - Non-treated	3.6 ± 0.2	5.3 ± 0.3**	8.0 ± 0.3**	5.2 ± 0.1**	2.8 ± 0.3**	2.0 ± 0.2
- 2,6-DHA treated	3.4 ± 0.2	5.4 ± 0.4**	8.0 ± 0.2**	5.2 ± 0.2**	2.8 ± 0.2**	1.9 ± 0.2

Values are means ± SEM obtained from 10 animals.

** $P<0.001$ significant difference when compare with normal control. whereas in hypercholesterolemic ones in both 2,6 DHA treated and non-treated were similar approximately 8.0 ± 0.3 mg/g liver

Table 8. Liver weight, cholesterol and triglyceride contents in liver of normal control animal.

No	Liver weight (g/100 kg BW)	Triglyceride (mg/g liver)	Liver cholesterol (mg/g liver)			free :Esterified
			Total	Free form	Ester form	
1	3.4	4.8	1.9	1.0	0.9	1.1
2	3.5	4.2	2.3	1.5	0.8	1.9
3	2.9	4.1	1.8	1.3	0.5	2.6
4	3.1	4.5	2.0	1.5	0.5	3.0
5	3.2	4.8	1.6	1.2	0.4	3.0
6	3.6	3.2	3.4	2.4	1.0	2.4
7	3.2	2.5	3.5	2.3	1.2	1.9
8	3.6	4.0	3.2	2.3	0.9	2.6
9	3.2	3.1	2.4	1.7	0.7	2.4
10	3.0	2.7	1.3	1.0	0.3	3.3
Mean ±SEM	3.3 ± 0.1	3.8 ± 0.3	2.3 ± 0.2	1.6 ± 0.2	0.7 ± 0.1	2.4 ± 0.2

Table 9. Liver weight, cholesterol and triglyceride contents in liver of hypercholesterolemic hamsters.

No	Liver weight (g/100 kg BW)	Triglyceride (mg/g liver)	Liver cholesterol (mg/g liver)			Free :Esterified
			Total	Free form	Ester form	
1	3.5	4.7	7.0	4.4	2.6	1.7
2	3.3	4.1	7.5	5.1	2.4	2.2
3	3.5	6.2	7.7	5.4	2.3	2.3
4	4.7	6.7	7.6	5.6	2.0	2.8
5	3.8	5.7	8.3	4.7	3.6	1.3
6	3.6	5.9	7.0	5.4	1.6	3.3
7	3.4	4.9	8.5	5.8	2.6	2.2
8	2.9	5.8	9.9	5.1	4.9	1.1
9	3.8	4.2	8.5	5.4	3.1	1.7
10	4.0	5.0	8.3	5.0	3.3	1.5
Mean ± SEM	3.6 ± 0.2	5.3 ± 0.3	8.0 ± 0.3	5.2 ± 0.1	2.8 ± 0.3	2.0 ± 0.2

Table 10. Liver weight, cholesterol and triglyceride contents in liver of 2,6-DHA (400 μmol/kg BW) supplemented to hypercholesterolemic hamsters for 7 days.

No	Liver weight (g/100 kg BW)	triglyceride (mg/g liver)	Liver cholesterol (mg/g liver)			free :Esterified
			Total	Free form	Ester form	
1	2.5	5.3	8.6	5.2	3.4	1.5
2	3.2	7.1	8.1	5.4	2.7	2.0
3	2.7	4.1	7.1	5.0	2.1	2.4
4	4.1	4.0	6.3	4.3	2.0	2.2
5	3.5	6.0	8.5	5.5	3.0	1.8
6	3.6	4.0	8.2	5.1	3.1	1.6
7	4.0	5.6	8.1	4.7	3.4	1.4
8	3.8	5.5	7.9	4.7	3.2	1.5
9	4.1	4.9	8.1	5.8	2.3	2.5
10	2.5	7.4	9.0	6.3	2.7	2.3
Mean ± SEM	3.4 ± 0.2	5.4 ± 0.4	8.0 ± 0.2	5.2 ± 0.2	2.8 ± 0.2	1.9 ± 0 1

Most of cholesterol in liver of hamster was in the free form. Hepatic free cholesterol contents in normal control was 1.6 ± 0.2 mg/g liver and increased to approximately 5 mg/g liver in hypercholesterolemic hamsters in both non-treated and 2,6-DHA treated group. The ester form of cholesterol in normal control was 0.7 ± 0.1 mg/g liver and was approximately 2.8 ± 0.3 mg/g liver and 2.8 ± 0.2 mg/g liver in non-treated and treated groups respectively. The ratio of free to esterified cholesterol in normal control was 2.4 ± 0.2 mg/g liver whereas it was lower (approximately 2) in livers of hypercholesterolemic animals with and without 2,6-DHA treated (Table 7). Liver weight, triglyceride and cholesterol contents of each animal in normal, hypercholesterolemic control and 2,6-DHA treated were shown in Table 8, 9 and 10 respectively.

5. Effect of 2,6-DHA on the distribution of cholesterol in plasma lipoproteins in hypercholesterolemic hamsters

This experiment was designed to determine the effect of 2,6-DHA on the distribution of cholesterol in various fractions of lipoproteins (VLDL, LDL and HDL). From the previous experiment, 2,6-DHA showed hypocholesterolemic action, the possible mechanism of 2,6-DHA to reduce plasma cholesterol may be related to decreased secretion of cholesterol from liver in the form of VLDL or increased uptake of cholesterol to liver in the form of VLDL and LDL for degradation. In this experiment, blood from overnight fasting hamsters were withdrawn from the abdominal aorta of three groups of animals, treated with 2,6-DHA at dose 400 μmol/kg BW (7 days), non-treated (control) and normal control (no cholesterol supplement) were used

to prepare plasma lipoproteins (VLDL, LDL and HDL) by sequential density gradient ultracentrifugation. Thereafter, plasma cholesterol in each fraction of lipoproteins were determined.

Table 11 and Figure 8 show distribution of cholesterol in VLDL, LDL and HDL fractions in normal control, chol-fed animals with and without 2,6-DHA treated at dose 400 μmol/kg BW. In normal control, total plasma cholesterol was approximately 124.2±4.3 mg/dl which distributed in VLDL, LDL and HDL approximately 8%, 15% and 77% respectively. In hypercholesterolemic hamsters without 2,6-DHA treated, plasma cholesterol was elevated to 392.0±19.6 mg/dl and was found to increase in VLDL and LDL fractions (136.0±10.6 mg/dl and 162.8±8.5 mg/dl respectively). The concentration of cholesterol in HDL was 93.3±3.7 mg/dl, which was not significantly different from that of normal control group (Table 11). In contrast 2,6-DHA treated on hypercholesterolemic hamsters resulted in a marked decrease in total plasma cholesterol which decreased in VLDL and LDL fractions but not in HDL fraction. The cholesterol concentration in VLDL and LDL fractions were highly significant difference from the non-treated group. The cholesterol concentration in total plasma, VLDL and LDL of 2,6-DHA treated were 176.2±4.6 mg/dl, 35.2±2.6 mg/dl and 45.9±2.2 mg/dl, respectively whereas cholesterol in HDL was 95.2±3.7 mg/dl which was not significant difference from the non-treated animal (Table 11). Table 12-14 showed distribution of cholesterol in plasma lipoprotein of each animal in normal control, hypercholesterolemic control and 2,6-DHA treated groups respectively.

The result in this experiment indicated that 2,6-DHA could reduce cholesterol in VLDL and LDL fractions but had no effect on HDL-cholesterol.

Table 11. Effect of 2,6-DHA (400 μmol/kg BW) supplemented for 7 days on distribution of cholesterol in plasma lipoproteins of hypercholesterolemic hamsters.

Group	Cholesterol (mg/dl)			
	Total	VLDL	LDL	HDL
Normal control	124.2 ± 4.3	9.6 ± 0.5	18.4±0.4	96.3±4.2
Hypercholesterolemic - Non-treated	392.0 ± 19.6^{++}	136.0 ± 10.6^{++}	162.8±8.5^{++}	93.3±3.7
- 2,6 DHA treated	176.2 ± 4.6**$^{+}$	35.2 ± 2.6**$^{++}$	45.9±2.2**$^{++}$	95.2±3.7

Values are means ± SEM obtained from 10 animals.

** P<0.001 significant difference from hypercholesterolemic (non-treated) control.

$^{+}$P<0.05, $^{++}$ P<0.001 significant difference from normal control.

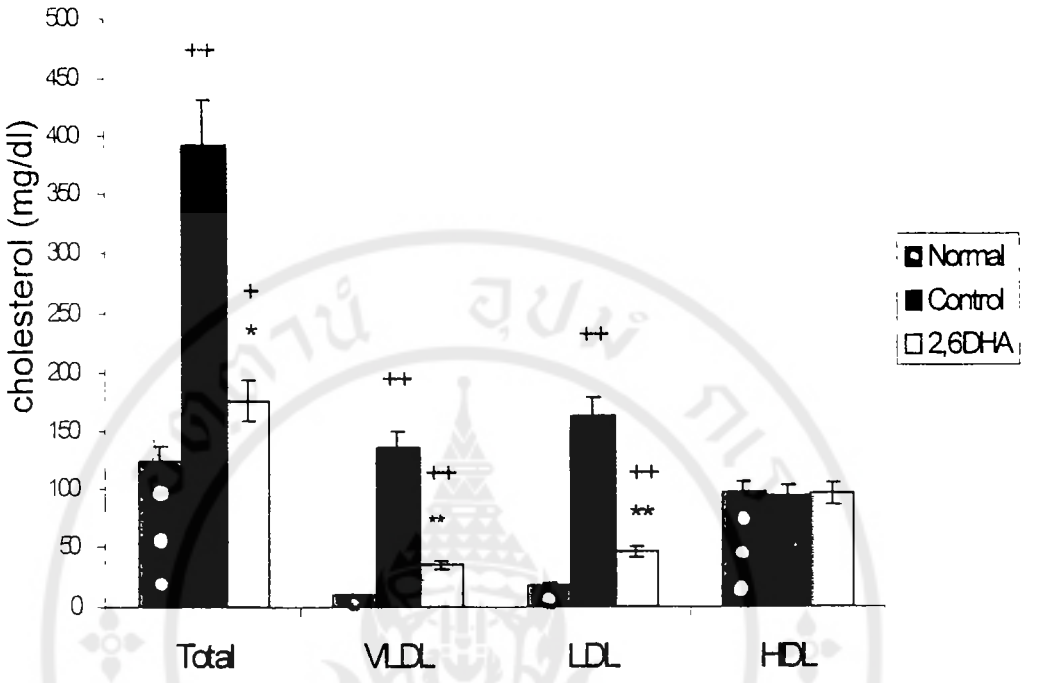


Figure 8. Distribution of cholesterol in plasma lipoproteins of hamster supplemented with 2,6-DHA 400μmol/kg BW for 7 days compared with hypercholesterolemic control and normal hamster.

Values are mean ± SEM obtained from 10 animals.

**P<0.001 significant difference from hypercholesterolemic (non-treated) control.

++P<0.001 significant difference from normal.

Table 12. Distribution of cholesterol in plasma lipoproteins of normal control animals.

No	Cholesterol (mg/dl)			
	Total	VLDL	LDL	HDL
1	151.3	8.1	17.8	125.6
2	130.7	11.2	16.3	103.2
3	110.9	8.1	17.3	85.4
4	112.7	8.9	19.6	84.2
5	139.1	12.9	18.8	107.5
6	124.9	9.0	18.4	97.5
7	112.7	8.5	17.0	87.2
8	112.7	8.5	20.4	83.7
9	131.0	11.3	20.5	99.3
10	116.2	9.0	18.0	89.3
Mean ± SEM	124.2 ± 4.3	9.6 ± 0.5	18.4 ± 0.4	96.3 ± 4.2

Table 13. Distribution of cholesterol in plasma lipoproteins of hypercholestrolemic hamsters (control animals).

No	Cholesterol (mg/dl)			
	Total	VLDL	LDL	HDL
1	484.4	146.4	215.1	123.1
2	470.4	182.5	199.0	88.9
3	447.4	184.5	172.4	90.5
4	366.0	120.5	150.3	95.2
5	427.3	172.4	165.4	89.5
6	331.3	100.5	150.4	80.4
7	317.9	102.4	130.2	85.2
8	361.5	119.7	148.6	93.2
9	323.5	100.3	136.0	87.2
10	391.3	130.7	160.4	100.2
Mean±SEM	329.1±19.9	136.0±10.6	162.8±8.5	93.3 ±3.7

Table 14. Distribution of cholesterol in plasma lipoproteins of 2,6-DHA (400 μmol/kg BW) supplemented to hypercholesterolemic hamsters for 7 days.

No	Cholesterol (mg/dl)			
	Total	VLDL	LDL	HDL
1	175.7	20.5	35.4	119.9
2	172.7	33.8	39.3	99.7
3	192.6	33.8	49.2	109.7
4	198.2	50.7	54.3	93.2
5	178.1	33.7	48.7	95.7
6	161.7	28.9	47.7	85.2
7	153.8	30.8	37.7	85.3
8	188.5	41.8	55.1	91.7
9	180.7	41.7	50.8	88.2
10	160.2	35.8	40.5	83.8
Mean ± SEM	176.2 ± 4.6	35.2 ± 2.6	45.9 ± 2.2	95.2 ± 3.7

6. Effect of 2,6-DHA on cholesterol 7∝-hydroxylase activity in hypercholesterolemic hamsters

This experiment was designed to evaluate the effect of 2,6-DHA on activity of cholesterol 7∝-hydroxylase which was the key enzyme for bile acid synthesis. The hypo-cholesterolemic action of 2,6-DHA on reducing plasma cholesterol in hypercholesterolemic hamsters as shown in the previous experiment may be related to the increased cholesterol 7∝-hydroxylase activity which is the key enzyme for conversion of cholesterol to bile acid for excretion. In this experiment, the liver of animals treated with 2,6-DHA at dose 400 μ mol/kg BW for 7 days and non-treated group (control) were used to prepare liver microsome for analysis of cholesterol 7∝-hydroxylase activity.

Table 15 shows the effect of 2,6-DHA (400 μmol/kg BW) treated for 7 days on cholesterol 7∝-hydroxylase activity and endogenous cholesterol content in liver microsome of hypercholesterolemic hamsters. The endogenous cholesterol content in liver microsome of both non-treated group (control) and treated group were not significantly different ($p>0.1$). It was 19.3 ± 0.9 and 21.6 ± 0.3 mg/g total lipid in non-treated group and 2,6-DHA treated groups, respectively.

In this study, cholesterol 7∝-hydroxylase activity was expressed as % conversion of [4^{14}-C] cholesterol to 7∝-hydroxy cholesterol per 30 min per mg protein. The result in Table 15 showed that, hypercholesterolemic hamsters continued on feeding cholesterol (0.2% BW) when supplemented with 2,6-DHA at dose 400 μmol/kg BW (treated group) showed a significant increase in cholesterol 7∝-hydroxylase activity as compared to hypercholesterolemic hamsters fed cholesterol (0.2% BW) without given

Table 15. Effect of 2,6-DHA (400 μmol/kg BW) supplemented to hypercholesterolemic hamsters for 7 days on endogenous cholesterol content and cholesterol 7α–hydroxylase activity in liver microsome.

Group	Endogenous cholesterol (mg/g total lipid)	Cholesterol 7α-hydroxylare activity (%conversion/mg prot/30 min)
Hypercholesterolemic		
- Non-treated	19.3 ± 0.9 (9)	0.5 ± 0.1 (7)
- 2,6-DHA treated	21.6 ± 0.3 (10)	3.4 ± 0.1** (8)

Values are means ± SEM

Number in parentheses are number of hamsters.

** P<0.001 significant difference when compare with hypercholesterolemic (non-treated) control.

Table 16. Endogenous cholesterol content and cholesterol 7α–hydroxylate activity in liver microsome of hypercholesterolemic hamsters.

No	Endogenous cholesterol (mg/g total lipid)	Cholesterol 7α-hydroxylare activity (%conversion/mg prot / 30 min)
1	21.1	0.5
2	18.1	0.5
3	15.9	0.7
4	17.8	0.5
5	22.7	0.4
6	19.7	0.4
7	20.8	0.4
8	22.2	ND
9	15.2	ND
Mean±SEM	19.3 ± 0.9	0.5 ± 0.1

ND = Not determined.

Table 17. Endogenous cholesterol content and cholesterol 7α–hydroxylate activity in liver microsome of 2,6-DHA 400 μmol/kg BW supplemented to hypercholesterolemic hamsters for 7 days.

No	Endogenous cholesterol (mg/g total lipid)	Cholesterol 7α-hydroxylare activity (%conversion/mg prot/ 30 min)
1	20.6	3.3
2	21.4	3.0
3	18.7	3.5
4	25.9	3.2
5	18.6	4.3
6	26.4	3.2
7	23.5	3.3
8	25.2	3.7
9	17.2	ND
10	20.9	ND
Mean ± SEM	21.6 ± 0.3	3.4 ± 0.1

ND = Not determined.

2,6-DHA (non-treated group). The cholesterol 7∝-hydroxylase activity of liver microsome from non-treated group was 0.5 ± 0.1% and was increased approximately 7 fold in 2,6-DHA treated group. Both groups of hamsters were sacrificed at similar time of day (08.00-10.00 a.m.) to avoid variation of the enzyme activity due to circadian rhythm. The endogenous cholesterol and cholesterol 7α-hydroxylase activity in liver microsomes of each animal in non-treated and 2,6-DHA treated groups were shown in Tables 16 and 17 respectively.

The result in this experiment indicated that hypocholesterolemic action of 2,6-DHA was probably due to an increase in the activity of cholesterol 7∝-hydroxylase which was the key enzyme regulated the conversion of cholesterol to bile acid for excretion.

7. Effect of 2,6 DHA on fecal cholesterol and bile acid excretion

Cholesterol could be eliminated out of the body in the form of unmodified cholesterol or was converted to bile acid in bile and excreted into feces. Therefore, this experiment was conducted to investigate the hypocholesterolemic effect of 2,6-DHA on cholesterol and bile acid excretion in feces of hamsters treated with 2,6-DHA at dose 400 μmol/kg BW for 7 days, non-treated (control) and normal control.

Table 18 and Figure 9 show effect of 2,6-DHA (400 μmol/kg BW) treatment for 7 days on fecal cholesterol content in normal control and hypercholesterolemic hamsters. Fecal cholesterol content in normal control was approximately 0.2 ± 0.01 mg chol/100 mg feces whereas in hypercholesterolemic hamsters there were significant higher in fecal cholesterol output to 1.3 ± 0.04 mg chol/100 mg feces and 7.3 ± 0.27 mg chol/100 mg

Table 18. Effect of 2,6-DHA 400 μmol/kg BW supplemented for 7 days on fecal cholesterol excretion.

Group	Body weight (g)	Fecal cholesterol (mg chol/100 mg feces)
Normal control (13)	86.9 ± 1.2	0.2 ± 0.01
Hypercholesterolemic - Non-treated (18)	85.8 ± 4.0	1.3 ± 0.04^{++}
- 2,6-DHA treated (10)	87.7 ± 1.0	7.3 ± 0.27**$^{++}$

Values are means ± SEM

Number in parentheses are numbers of hamsters.

** P<0.001 significant difference when compare with hypercholesterolemic (non-treated) control.

$^{++}$ P<0.001 significant difference when compare with normal control.

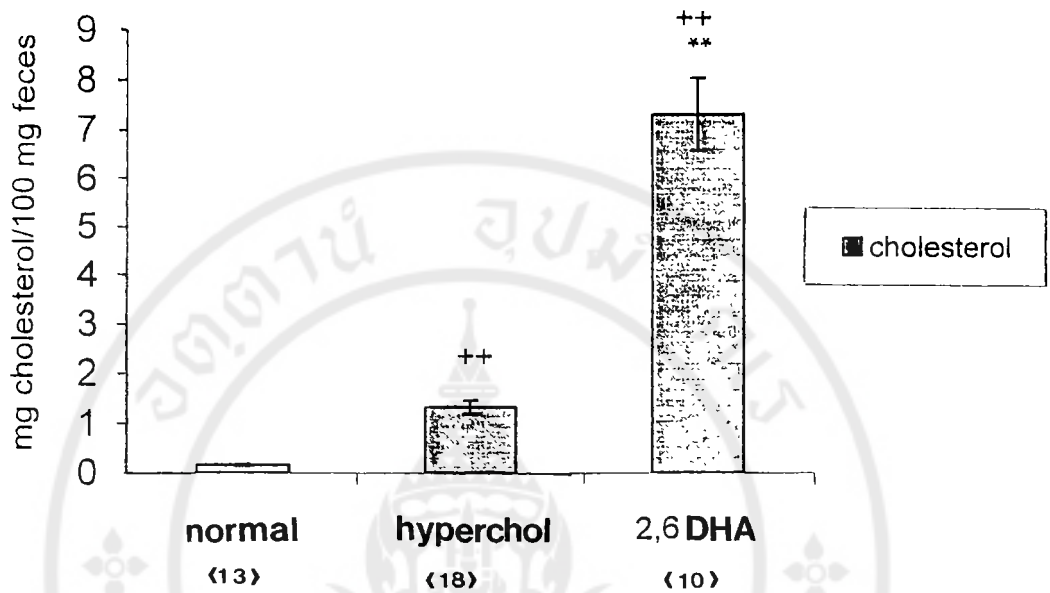


Figure 9. Fecal cholesterol excretion of hamster supplemented with 2,6-DHA 400 μmol/kg. BW for 7 days compared with hypercholesterolemic control and normal control.

Values are means ± SEM obtained from 10 animals.

Number in parentheses are numbers of hamsters.

**P<0.001 significant difference from hypercholesterolemic (non-treated) control

++P<0.001 significant difference from normal control.

Table 19. Body weight and fecal cholesterol excretion (mg/100 mg feces) of normal control, hypercholesterolemic control, and 2,6-DHA 400 μmol/kg BW supplemented animals for 7 days.

No	Normal		Hypercholesterolemic		Hyperchol + 2,6-DHA	
	Body wt (g)	Cholesterol	Body wt (g)	Cholesterol	Body wt (g)	Cholesterol
1	87.5	0.2	90.4	1.4	91.4	5.9
2	84.3	0.2	89.5	1.4	80.5	8.3
3	92.4	0.2	88.4	1.1	89.2	9.3
4	88.5	0.2	88.2	1.2	84.9	7.4
5	80.8	0.2	83.3	1.3	88.7	7 7
6	82.4	0.2	87.6	1.5	88.1	7.5
7	93.2	0.1	86.3	1.3	87.8	6.3
8	87.8	0.1	80.9	1.4	91.3	7.8
9	82.3	0.2	86.1	1.4	92.1	7.1
10	84.7	0.2	94.7	1.3	84.2	6.7
11	93.8	0.2	82.0	1.4	85.6	5.6
12	87.2	0.2	87.5	1.5	88.9	7.2
13	86.0	0.1	80.5	0.9	87.5	6.7
14			87.1	1.3	86.4	9.4
15			83.0	1.4	89.1	3.5
16			80.2	1.6	90.2	7.9
17			86.9	1.3	86.5	6.9
18			81.2	1.6	87.7	7.4
19					89.4	7.3
20					91.5	8.4
21					90.8	7.4
22					92.6	6.7
23					84.2	8.2
24					90.1	9.1
Mean ±SEM	87.0 ±1.2	0.2 ±0.0	85.8 ±0.9	1.3 ±0.0	88.3 ± 0.6	7.3 ±0.3

feces in non-treated and 2,6-DHA treated groups, respectively. It could be seen that treatment of 2,6-DHA caused a significant increase in excretion of cholesterol into feces when compared to the non-treated group. The body weight and cholesterol content in feces of each animal in normal, hypercholesterolemic control and 2,6-DHA treated were shown in Table 19.

Table 20 and Figure 10 show effect of 2,6-DHA (400 μmol/kg BW) treatment for 7 days on fecal bile acid excretion of normal and hypercholesterolemic hamsters. In normal control, fecal bile acid was to 2.9±0.1 mmol/100 mg feces whereas in chol-fed group, treated and non-treated with 2,6-DHA at dose 400 μmol/kg BW showed significant increase in fecal bile acid to 6.7±0.4 and 8.9±0.1 mmol/100 mg feces respectively. The fecal bile acid in the treated group was significantly higher ($p<0.01$) than the non-treated group.

The result in this experiment indicated that 2,6-DHA increased bile acid excretion into feces. The body weight and bile acid content in feces of each animal in normal, hypercholesterolemic control and 2,6=DHA treated were shown in Table 21.

Table 20. Effect of 2,6-DHA (400 μmol/kg BW) supplemented for 7 days on fecal bile acid excretion.

Groups	Body weight (g)	Bile acid concentration (mmol/100 mg feces)
Normal control (5)	87.4 ± 0.4	2.9 ± 0.1
Hypercholesterolemic		
- Non treated (4)	87.7 ± 0.3	6.7 ± 0.4
- 2,6-DHA treated (8)	88.2 ± 0.5	8.9 ± 0.3**$^{++}$

Values are means ± SEM

Number in parentheses are number of hamster

** P<0.001 significant difference when compare with hypercholesterolemic (non-treated) control.

$^{++}$ P<0.001 significant difference when compare with normal control.

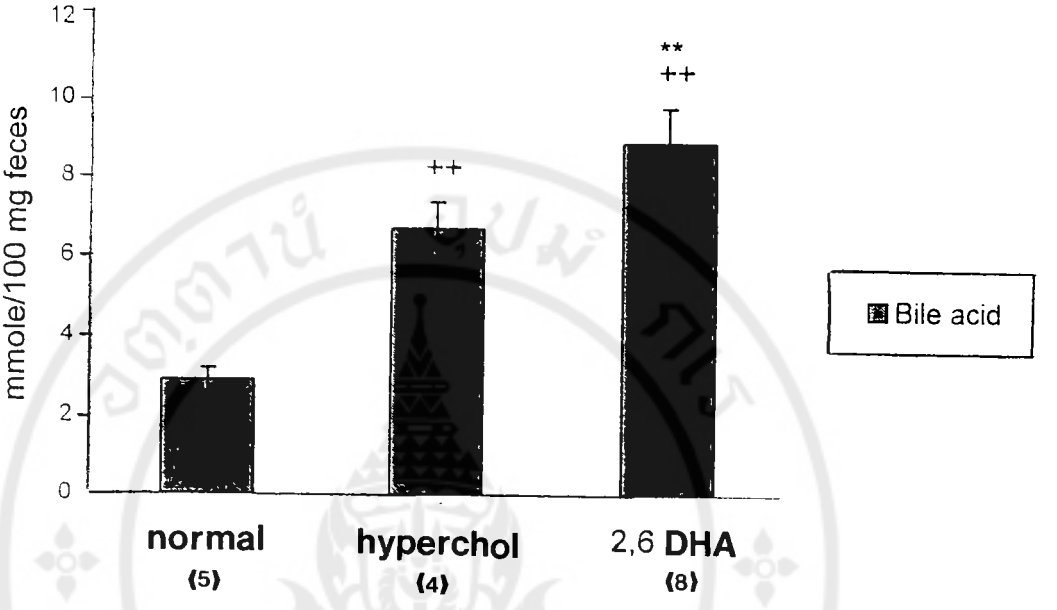


Figure 10. Fecal bile acid excretion of hypercholesterolemic hamster supplemented with 2,6 DHA 400 μmole/kg BW for 7 days compared with control and normal hamsters.

Values are means ± SEM obtained from 10 animals.

Number in parentheses are numbers of hamsters.

**P<0.001 significant difference from hypercholesterolemic control.

++P<0.001 significant difference from normal control.

Table 21. Body weight and fecal bile acid excretion of normal control, hypercholesterolemic control and 2,6-DHA (400 μmol/kg BW) supplemented animals for 7 days.

No	Normal		Hypercholesterolemic		Hyperchol+2,6-DHA	
	Body wt (g)	Bile acid	Body wt (g)	Bile acid	Body wt (g)	Bile acid
1	87.5	2.7	88.4	5.9	89.2	8.4
2	88.5	3.3	87.6	6.4	88.4	8.4
3	87.8	2.8	87.5	7.5	90.1	8.2
4	87.2	2.9	87.1	7.1	86.5	9.1
5	86.0	2.9			88.2	9.0
6					87.0	9.0
7					86.5	8.6
8					84.2	8.4
Mean ±SEM	87.4 ± 0.4	2.9 ± 0.1	87.7 ± 0.3	6.7 ± 0.4	87.5 ± 0.7	8.6 ± 0.1

CHAPTER IV

DISCUSSION

Among various animal species, hamster is commonly used as an experimental animal model for the study of lipid metabolism because of it similarity with that of human (92,95). It is considered to be more sensitive for induction of hypercholesterolemia than rat which is able to adapt to large fluctuation in dietary cholesterol with little change in plasma cholesterol concentration (93). Rat is an animal model that differs significantly from humans with respect to cholesterol and lipoprotein metabolism (93,94). In contrast to the rat, the hamster more closely resembles humans with respect to basal rates of cholesterol and bile acid synthesis (92), composition of bile acid excretion (92,94), various aspects of lipoprotein transport (92,93) and response to dietary and pharmacologic treatment (93,95). It is rather difficult to induce hypercholesterolemia in rat by feeding just high cholesterol diet because the rat has a high efficient mechanism for converting excess cholesterol into bile acid (93). Furthermore, in the rat biliary cholesterol secretion is tightly coupled to bile acid and phospholipid out put and many dietary and pharmacologic treatment that disturb this coupling process in human and other animals are without effect in the rat (117). In contrast, the hamster manifests much lower rate of whole-body and hepatic cholesterol metabolism than the rat and response quickly to a variety of pharmacologic treatment. For these reasons, in this study the hamster was chosen as an experimental animal model to investigate the hypolipidemic action of 2,6-DHA in the hyperlipidemic state.

In the present study, hypercholesterolemic hamsters were obtained by supplementing cholesterol at 0.2% BW to male hamsters fed on chow diet for 3 weeks. During this period these hamsters gained body weight approximately 10% similar to those with no cholesterol supplement (Table 1). Moreover, hamsters with or without cholesterol supplement showed normal behavior and there were no sign of sickness. This indicated that our experimental animals were not suffered from feeding high cholesterol at this level and were healthy for further treatment with 2,6-DHA.

Hamsters supplemented with cholesterol at 0.2% BW showed a significant increase in plasma cholesterol (Figure 2). Within 3 weeks, plasma cholesterol was increased to approximately 300 mg/dl (Table 2). In contrast, in the early study, feeding cholesterol at similar level to rat for 3 weeks could not develop hypercholesterolemia (data was not shown). This observation was consistent to that observed previously by Sessions et al (94) that addition of cholesterol to diet of hamsters cause a dose dependent increase in serum cholesterol and triglyceride concentration. In addition, Horton et al (93) found that hamsters were much more sensitive than rat to dietary cholesterol content because the latter had higher basal level of cholesterol 7α-hydroxylase expression which allow them to convert excess dietary cholesterol to bile acid faster than hamsters (93).

Several mechanisms namely absorption of dietary cholesterol, the hepatic conversion of cholesterol to bile acid, the feedback inhibition of endogenous cholesterol synthesis and the regulation of LDL receptor pathway may involve in causing hypercholesterolemia in hamster by cholesterol feeding. Horton et al (93) reported that hepatic cholesterol synthesis was suppressed by more than 90% in response to increasing loads of dietary cholesterol. In addition, Shimomera et al (96) found that in

hamster cholesterol, feeding reduced the mRNA encoding multiple enzyme in cholesterol biosynthetic pathway, mainly HMG-CoA reductase which is the key enzyme for cholesterol synthesis. Moreover the mRNA for the low density lipoprotein (LDL) receptor was also reduced by cholesterol feeding (96). In hamster, the basal level of cholesterol 7α-hydroxylase expression was very low and the expression was not induced by dietary cholesterol (93). These possibilities mentioned may account for the development of hypercholesterolemia in cholesterol-fed hamsters observed in this study.

In association with the development of hypercholesterolemia, hamster fed high cholesterol diet also developed hypertriglyceridemia (Table 3). This observation was consistent to that reported in other studies that feeding a high cholesterol diet to hamsters caused dose-dependent increases in both plasma cholesterol and triglyceride (97,98). Session et al (98) found that cholesterol feeding was associated with an increase in the activity of hepatic phosphatidate phosphohydrolase 1 (PAP-1) which is a key enzyme in regulating triacylglycerol synthesis. As the consequence, triacylglycerol synthesis was increased and subsequently increased VLDL secretion. Since VLDL particle is generally consisted of both triacylglycerol and cholesterol in its hydrophobic core, therefore there was an absolute requirement of cholesterol for synthesis and secretion of VLDL (98). The dietary cholesterol induced hypertriglyceridemia and hypercholesterolemia were accompanied by increase in VLDL and LDL whereas the cholesterol in HDL might (99) or might not be change (92).

In this study, within 3 weeks of cholesterol feeding the marked increase in total plasma cholesterol was associated with elevation of cholesterol in VLDL and LDL but not in HDL-fraction (Table 13). This finding is consistent to that reported by Jackson et

al (99) and Fungwe et al (92). They showed that the increase in VLDL and LDL in cholesterol fed hamster was due to increased biosynthesis of apoB and secretion of VLDL. The increase in VLDL secretion may contribute to the increase in LDL formation from VLDL in plasma. The elevated LDL level was probably primarily a result of down regulation of cholesterol to the liver results in decreased receptor mediated uptake of LDL (98).

In the present study, 2,6-DHA an analogue of 2,4,6-THA which is aglycone part of a naturally occurring glucoside from a medicinal plant namely *Curcuma comosa* was studied in comparison with 2,4,6-THA on its potency as hypolipidemic agent. It was shown clearly that 2,6-DHA at dose of 300-600 μmol/kg BW could lower both cholesterol and triglyceride in plasma of hypercholesterolemic hamsters in a dose-dependent manner within 7 days (Table 3). The maximum dose attained was 600 μmol/kg BW because at a high dose (800 μmol/kg BW) a lesser effect was observed. Similar dose response effect was also observed in the case of 2,4,6-THA in the parallel study (100). It is possible that 2,6-DHA as well as 2,4,6-THA at 800 μmol/kg BW in the administered solvent might precipitate in the gastrointestinal tract resulted in incomplete absorption. It was observed that some hamsters treated with 2,6-DHA or 2,4,6-THA at 800 μmol/kg BW had diarrhea and loss weight. Therefore, it was considered that 2,6-DHA and 2,4,6-THA at 800 μmol/kg BW dose was not suitable and might cause the animal loss appetite resulting in body weight loss. However, all doses of 2,6-DHA use in this study (300-800 μmol/kg BW) were not toxic to hamsters because most of them were healthy throughout the experiment. At the time of sacrifice, the appearance and weight of liver were similar to the normal control hamsters. The

highest dose of 2,6-DHA used in this study (800 μmol/kg BW) was approximately 20 times below LD_{50} of 2,4,6-THA (15,847 μmol/kg BW) (data was not shown).

The effective dose of 2,6-DHA in lowering plasma lipids chosen to study its mechanism of action was 400 μmol/kg BW because at the higher dose (600 μmol/kg BW), only small amounts of cholesterol and triglycerides were decreased (Table 4,5, Figure 3,4). Therefore it is more practical to use the effective lower dose and prolong the time of treatment. The maximum lipid lowering effect of 2,6-DHA at 400 μmol/kg BW was attained after 10 days of treatment (Table 6, Figure 6,7). Prolong the treatment to 14 days showed no significant difference in plasma cholesterol and triglyceride levels. This observation was similar to that observed in the case of 2,4,6-THA in other study (100), suggesting that the maximum effect of both drugs had attained or the reduced cholesterol and triglyceride already reach the normal levels of hamsters. The result indicated that 2,6-DHA was as effective as 2,4,6-THA in lowering plasma lipids in hyperlipidemic hamsters.

Hamsters fed high cholesterol diet showed increase in liver cholesterol and triglyceride. As shown in Table 7, the liver cholesterol content was increased 3.5 fold whereas the triglyceride content slightly increased (1.4 fold) when compared with hamsters fed on normal diet without cholesterol supplement. These observation was consistent with those reported earlier (92,96,101,102) that feeding a high cholesterol diet to hamsters markedly increased liver cholesterol content. A marked increase in total cholesterol was apparently increase in both free (3.3 fold) and ester forms (4 fold). However, when the ratio of free to esterified forms of cholesterol was considered, there was a decrease in this ratio (2.0±0.2) when compared to that of the normal control

hamsters (2.4±0.2) (Table 7). This finding indicated that there was an increase in the ester form of cholesterol in hamsters fed high cholesterol diet. The result, indirectly indicated that cholesterol feeding increased the activity of acyl-CoA cholesterol acyltranferase (ACAT) located in the endoplasmic reticulum and function to esterify free cholesterol for storage. The increase in ACAT activity in response to the high cholesterol diet observed in this study was in agreement with that previous reported by Jackson et al (99) and Ochoa et al (103) who found that administration of diet containing 2% cholesterol to hamsters resulted in an increase ACAT activity. A slight increase in liver triglyceride of cholesterol-fed hamsters indicated that these hamsters efficiently excreted excess triglyceride into blood stream in the form of VLDL and this contributed to a significant increase in plasma triglyceride and VLDL observed in this study. Although administration of 2,6-DHA to hyperlipidimic hamsters led to a significant decrease in both plasma cholesterol and triglyceride (Figure 6,7) but in liver there was no change in both lipids when compared to the corresponding control group (Table 7). The result indicated that the hypolipidemic action of 2,6-DHA was not due to accumulation of lipids in the liver because there was no significant difference in liver weight and lipid content between 2,6-DHA treated and non-treated group (Table 7). Moreover, no change in the ratio of free to ester form of cholesterol indicated that 2,6-DHA had no effect on ACAT activity similar to that observed in the case of 2,4,6-THA in other study (100).

There are several possibilities which may involve in the hypolipidemic action of 2,6-DHA. Firstly, this compound may impair intestinal lipids absorption, resulted in lower plasma cholesterol and triglyceride. This possibility was not investigated in this

study but could not be excluded because there was a significant increase in fecal cholesterol excretion in the treated groups (Figure 9).

Secondly, 2,6-DHA may inhibit hepatic cholesterol synthesis especially on the activity of HMG CoA-reductase, the regulatory enzyme in cholesterol synthesis in liver similar to the action of drugs such as statin (97) and bezafibrate (105). Inhibition of hepatic cholesterol synthesis is known to be a potent way to reduce plasma cholesterol concentration by these drugs. However, in this study, HMG CoA reductase activity was not determined because the hypolipidemic action of 2,6-DHA was investigated in cholesterol feeding hamsters in which there was reported that the activity of HMG CoA reductase was inhibited by dietary cholesterol (103). In order to see the effect of 2,6-DHA on HMG CoA reductase activity, the study should be carried out in normolipidemic animal.

Thirdly, 2,6-DHA may cause upregulation of LDL receptor activity and consequently reduce plasma cholesterol. It has been shown in rats, that an increase in LDL receptor protein mass, for instance by induction of LDL receptor gene transcription by 17α-ethinyl estradiol, lead to a reduction in plasma cholesterol (97). In addition, cholesterol lowering drug such as HOE 402 was also shown to exert its lipid lowering effect by a direct activation on hepatic LDL receptor activity (97). Moreover, benzafibrate, another lipid lowering drug was also suggested to act by increasing LDL receptor activity as the result from its inhibitory effect on HMG CoA-reductase activity (104). In this study, the effect of 2,6 DHA on LDL receptor activity or LDL clearance rate was not determined but the finding that addition of 2,6-DHA to the cholesterol-fed hamsters caused a reduction in LDL-cholesterol (Table 11) may be a suggestive evidence that 2,6-DHA may stimulate LDL receptor activity.

Although the mechanism whereby 2,6-DHA stimulate LDL receptor activity has to be further investigated, two possible explanations should be taken into consideration. First, the LDL-receptor activity may be stimulated by 2,6-DHA at both transcriptional and translational level or through a change in the receptor mediated endocytotic pathway. As a consequence of either action, more LDL-receptor would be available on the cell surface, thus resulting in an increased LDL turnover. The net result would be that more cholesterol is taken up by the liver cell. The evidence that 2,6-DHA may increase LDL receptor activity came from its stimulatory effect on cholesterol ??-hydroxylase activity. Since it has been reported that augmentation of hepatic ??? hydroxylase expression by adenovirus-mediated transfer of gene encoding the enzyme into hamsters (67) and by cholestryranime treatment (67,107) could lead to increase LDL-receptor activity. The observation that there was no change in hepatic free and ester forms of cholesterol in 2,6-DHA treated when compared to the non-treated group (Table 7) suggested that cholesterol taken up via the LDL receptor pathway might be directed into the pool for biliary secretion rather than into the pool for cholesterol esterification. In agreement with this speculation, there was a report that in peritoneal macrophage, cholesterol uptake from LDL is a poor activator of ACAT (97). Besides a direct biliary cholesterol secretion, cholesterol uptake from LDL can serve as substrated for cholesterol 7α-hydroxylase, the rate limiting enzyme of bile acid synthesis. The excretion of cholesterol into bile and the conversion of cholesterol to bile acid may be the two routes that 2,6-DHA treated hamsters could maintain hepatic cholesterol indifferent from that of the non-treated group in cholesterol feeding condition and at the same time could reduce plasma cholesterol level.

Therefore, the fourth possible explanation for hypocholesterolemic effect of 2,6-DHA could be a stimulatory effect on conversion of hepatic cholesterol to bile acid. To investigate this possibility, the activity of cholesterol 7α-hydroxylase catalyzed the rate limiting step in bile acid synthesis was determined in liver microsomes of cholesterol-fed hamsters with and without 2,6 DHA treated for 7 days. As shown in Table 15, cholesterol 7α-hydroxylase activity in 2,6-DHA treated group was approximately 7 fold higher than that of the non-treated group. The observed increase in cholesterol 7α-hydroxylase activity is rather unlikely to be due to circadian rhythum of the enzyme because both groups of hamsters were sacrificed at a similar time of the day (8.00-10.00 a.m.). In addition, It has been shown that in hamsters, both cholesterol 7α-hydroxylase activity and mRNA level were similar at the mid-dark and mid-light point of the light cycle, suggesting that diurnal fluctuation in the enzyme activity is much less pronounced in the hamster than in rat (93). This is consistent with the finding that hamsters consume approximately as much food during the two phases of light cycle whereas rats eat exclusively during the dark phase of light cycle. Moreover, it may be possible that the stimulatory effect of 2,6-DHA on cholesterol 7α-hydroxylase activity may be due to a difference in endogenous cholesterol that affect specific activity of ^{14}C-cholesterol substrate and leads to different enzyme activity. This possibility can be eliminated because the endogenous cholesterol content in liver microsome of 2,6-DHA treated and non-treated were similar (Table 15).

The mechanism by which 2,6-DHA activates cholesterol 7α-hydroxylase activity is not known at present. Two mechanisms could account for an increase in cholesterol 7α-hydroxylase activity. Firstly, short term effect may involve phosphorylation/

dephosphorylation of the enzyme protein in which the active form of the enzyme is in the phosphorylated state (105). In this case, 2,6-DHA may inhibit phosphatase activity thus maintaining the enzyme in the phosphorylated form. To verify this possibility, the phosphorylated form of the enzyme should be found in 2,6-DHA treated liver microsome. At present, there is no evidence indicate that 2,6-DHA act by this mechanism.

Secondly, long term effect of 2,6-DHA on increasing cholesterol 7α-hydroxylase activity may involve the stimulation at transcriptional level leads to increases mRNA corresponding to cholesterol 7α-hydroxylase. By using specific antibodies towards the enzyme protein and a cDNA probe, Li et al (118) showed that in rats, treatment with cholestyramine and cholesterol increased the levels of enzyme protein and the corresponding mRNA. Further study should be done to measure the level of mRNA corresponding to cholesterol 7α-hydroxylase to see whether 2,6-DHA treatment could increase the mRNA level.

The stimulatory effect of 2,6-DHA on cholesterol 7α-hydroxylase activity may either be directly or secondary to the effect of 2,6-DHA on the increasing bile acid secretion. An increase in bile acid secretion could stimulate cholesterol 7α-hydroxylase activity as has been shown by Jelinek et al (105) that the level of mRNA for cholesterol 7α-hydroxylase increased when bile acids were depleted by dietary cholestyramine and decreased when bile acids were consumed. The finding reported in this study that there was an increase in bile acid excretion in feces of 2,6-DHA treated hamster comparing with the corresponding control is consistent to the observed increase in cholesterol 7α-hydroxylase activity.

The increased bile acid excretion in feces of 2,6-DHA treated hamsters suggested that 2,6-DHA may effect reabsorption of bile acid through enterohepatic circulation into liver. Although, this effect seems to be similar to that of cholestyramine (93,107) and other bile acid sequestrants (93,107) in increasing cholesterol 7α-hydroxylase but the action of 2,6-DHA on interruption of bile acid absorption via enterohepatic circulation may be different. In previous study (77), 2,6-DHA and well as 2,4,6-THA were rapidly and readily absorbed after intraduodenal administration and also showed a dose-dependent stimulation on bile acid secretion (77). In this view, it is unlikely that 2,6-DHA mediates its action by sequestering cholesterol in the gastrointestinal tract in the same way as cholestyramine. However, it remained possible that 2,6-DHA may affect the formation of different bile acid species that interfere with the enterohepatic circulation of normal bile acid. This speculation is based on the finding that ursodeoxycholic acid lowered LDL cholesterol by interfering the enterohepatic circulation (108). In addition, change in the bile acid profile especially in the ratio of cholate:chenodeoxy cholate has been shown in hamster to affect solubility of cholesterol in bile acid micelle (95).

The finding that 2,6-DHA increased excretion of bile acid and cholesterol in feces is similar to that observed in the case of 2,4,6-THA in the parallel study (100). However the amount of cholesterol in feces of 2,6-DHA treated was slightly lower than that of 2,4,6-THA treated whereas the bile acid content was similar (100). The difference in cholesterol excretion by different analogue of acetophenone may be related to different hydrophobicity of each analogue that affect formation of different bile acid species and as the consequence affect solubility of cholesterol in bile acid

micelle. This speculation was based on the previous finding that various acetophenones had different choleretic activity (109).

The hypocholesterolemic effect of 2,6-DHA by increasing both excretion of cholesterol in feces and cholesterol 7α-hydroxylase activity made this compound very attractive as lipid lowering agent. Usually, cholesterol absorption correlates with plasma cholesterol level in some human population (93) and agents that interfere with cholesterol absorption have modest cholesterol–lowering activity (93). Hepatic cholesterol 7α-hydroxylase represents another promising target in lowering plasma cholesterol. It has been reported that adenovirus-mediated transfer of a gene encoding cholesterol 7α-hydroxylase into hamsters markedly increased cholesterol 7α-hydroxylase activity and rendered these animals became resistant to increasing plasma cholesterol upon consuming high cholesterol diet (119). The available bile acid sequestrants increase hepatic cholesterol 7α-hydroxylase expression at lesser extent and, as a consequence, have only modest cholesterol-lowering activity. The dual effect of 2,6-DHA on cholesterol absorption and activity of cholesterol 7α-hydroxylase activity made this compound an effective hypocholesterolemic agent. In the future more effective strategies for increasing cholesterol 7α-hydroxylase expression should be the prominent way in lowering plasma cholesterol level.

In our study, the effect of 2,6-DHA on decreasing triglyceride, VLDL and LDL-cholesterol was similar to the action of garlic (110,111), fibric acid derivatives (112) and nicotinic acid (113) which are lipid lowering agents. There are two possibilities that 2,6-DHA can reduce triglyceride, VLDL and LDL-cholesterol. Firstly, on reduction of VLDL secretion by causing abnormalities in appoprotein B-100. Mutation of apoB-100

since it may also increase LDL receptor activity thus leading to lowering plasma LDL cholesterol. The supporting evidence that increasing cholesterol 7α–hydroxylase activity was appear to be a condition which VLDL is not released from the liver resulting accumulation of lipid in liver (114). However, this possibility was unlikely because 2,6- DHA did not alter the lipid content in liver. Secondly, 2,6-DHA may increase catabolism of VLDL by increasing the lipoprotein lipase (LPL) activity (104) which is the enzyme for hydrolysis of triglyceride in VLDL particle to release free fatty acid and monoglycerides for oxidation by muscles and storage by adipose tissue (115). This possibility was consistent with the hypolipidemic action of benzafibrate that reduced plasma lipids by increasing the concentration of apo CII that is a cofactor for LPL (116). In the present study, the effect of 2,6-DHA on LPL activity could not be ascurtained. Further study on various factors affecting LPL activity and their interaction with 2,6-DHA will clarify this possibility.

Finally, base on the evidence obtained in this study, it was concluded that 2,6-DHA is an effective lipid lowering agent with similar potency to that of 2,4,6-THA. The main hypolipidemic action of 2,6-DHA was on activation of cholesterol 7α-hydroxylase activity and increased excretion of bile acid and cholesterol in feces. The increased fecal excretion of bile acid would up-regulated LDL receptors and lead to increase plasma clearance of VLDL and LDL. Further studies are required to clearify the exact mechanism of action of 2,6-DHA on cholesterol 7α-hydroxylase. However, the present result suggest that 2,6-DHA may have potential for development as a lipid lowering drug for treatment of hyperlipidemia.

CHAPTER V

SUMMARY

1. Hypolipidemic effect of 2,6-dihydroxyacetophenone (2,6-DHA) was studied in hypercholesterolemic hamsters induced by feeding cholesterol at 0.2% body weight for 3 weeks.
2. 2,6-DHA at 300-800 μmol/kg BW effectively lowered level of both plasma cholesterol and triglyceride in a dose dependent manner.
3. The minimum effective dose of 2,6-DHA in lowering plasma lipids was 400 μmol/kg BW which showed maximum effect after 10 days of treatment.
4. The hypolipidemic effect of 2,6-DHA was not accompanied by an increase in liver cholesterol and triglyceride contents suggesting that its effect was not due to accumulation of lipids in liver.
5. There was no change in free and ester forms of cholesterol in liver after 2,6-DHA treatment, suggesting that this compound had no effect on the activity of acyl CoA-cholesterol acyltranferase which catalyzes conversion of cholesterol to cholesteryl ester for storage.
6. Hamsters treated with 2,6-DHA at dose 400 μmol/kg BW for 7 days showed significant increase in the activity of cholesterol 7α-hydroxylase activity in liver microsome.
7. The hypolipidemic effect of 2,6-DHA was accompanied by an increase in excretion of cholesterol and bile acid in feces.

8. The cholesterol lowering effect of 2,6-DHA was related to decreased cholesterol in VLDL and LDL but not in HDL fractions.
9. It was concluded that the hypolipidemic effect of 2,6-DHA was related to its stimulatory effect on cholesterol 7α-hydroxylase activity and on increased excretion of cholesterol and bile acid.

Further studies are required to clarify the exact mechanism of action of 2,6-DHA on cholesterol 7α-hydroxylase and on cholesterol absorption in gastrointestinal tract, as well as on lipoprotein lipase activity in plasma.

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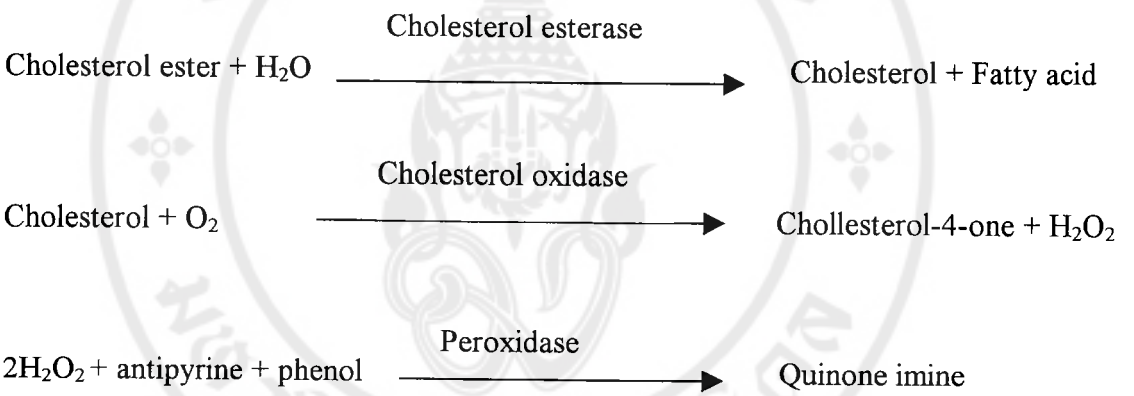
APPENDIX I

DETERMINATION OF TOTAL, UNESTERIFIED AND ESTERIFIED CHOLESTEROL

(Enzymatic method)

Principle



Reagents

1. Working enzyme reagent: This solution composes of three enzyme (cholesterol esterase, cholesterol oxidase and peroxidase) and antipyrine.
2. Buffer solution: This solution composes of phosphate and phenol.

Working Solution

Dissolve enzyme mixtures with volume of buffer solution, when they are completely dissolved, transfer to the same buffer bottle. This working solution should be used within 1 month after preparation.

Procedure for determination of total cholesterol

1. Pipette the solution as indicated below into a tube:

	Blank (ml)	Test (ml)
Working reagent	1.5	1.5
Serum, Liver homogenate(Sample)	-	0.01
Mix on a vortex, then incubate at 37°C for 10 min		

2. Read the absorbance at 550 nm vs. a blank

Calculation

Concentration of cholesterol in sample (mg/dl)

$$= \frac{\text{O. D. unknown x concentration of standard}}{\text{O. D. standard}}$$

Procedure for determination of unesterified cholesterol and esterified cholesterol

The unesterified cholesterol was determinated by a similar procedure of total cholesterol except cholesterol esterase in working enzyme reagent is omit

The esterified cholesterol was calculated for each sample according to the following formula:

Total cholesterol = unesterified cholesterol + esterified cholesterol

References:

1. Burstein M, Scholnick HR, Morfin R. Rapid method for the isolation of lipoprotein from human serum by precipitation with polyanions. J Lipid Res 1970; 11:583-597.
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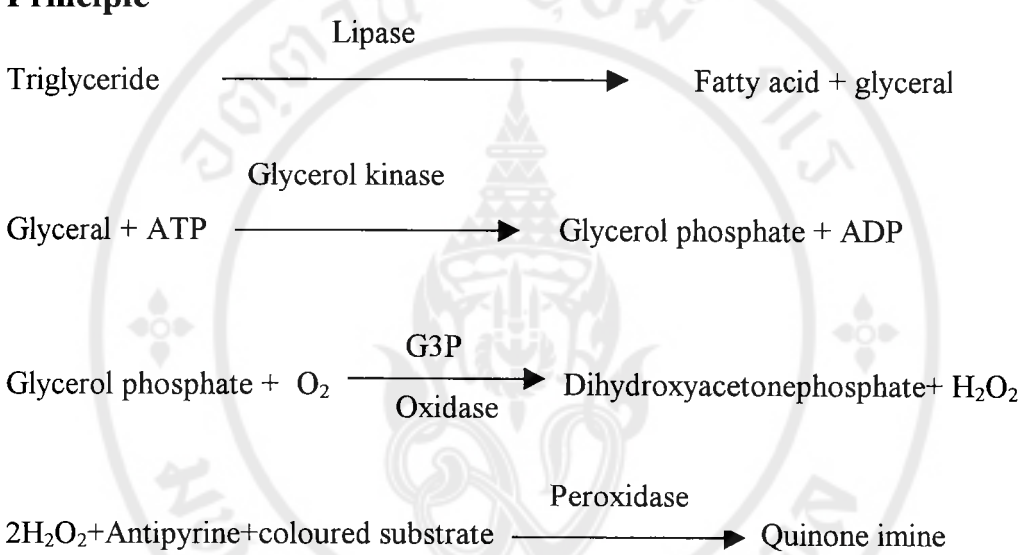
APPENDIX II

DETERMINATION OF TRIGLYCERIDE

(Enzymatic method)

Principle



Reagents

1. Enzyme Mixtured 10 bottles
2. Buffer solution 30 ml x 10 bottles
3. Standard Trilyceride 150,300 and 450 mg/dl

Stored all reagent at 2-8 ^{O}C

Working Solution

Dissolve enzyme mixtures with small volume of buffer solution, When they are completely dissolved, transfer to the same buffer bottle. This working solution should be used within 2 weeks after preparation.

Procedure

1. Add the solution into each tube as follows:

	Blank (ml)	Test (ml)
Working Solution	1.5	1.5
Serum (sample)	-	0.01

2. Mix, incubate at 37°C for 5 min
3. Read O. D. at 520 nm against reagent blank.

Calculation

Concentration of triglyceride in sample (mg/dl)

$$= \frac{\text{O.D. Unknown x Concentration of Standard}}{\text{O.D. Standard}}$$

References

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APPENDIX III

DETERMINATION OF BILE SALT

(Modified method of Turnberg, 1969)

Principle

Total bile acid content in feces was determinated by using 3α-hydroxysteroid dehydrogenase. The enzyme catalyzes specific oxidation of 3α-hydroxysteoid including all kinds of bile acids to ketone group by requiring NAD^+ as coenzyme. The amount of 3α-hydroxysteoid is then determined from the amount of NADH formed by measuring absorbance at 340 nm.

$$3\ \alpha\text{-hydroxysteroid} + NAD \xrightarrow{\text{3-HSD}} 3\ \text{keto-steroid} + NADH + H^+$$

Reagent and preparation

1. The standard bile salt

An aliquot of standard sodium taurocholate containing 0.025, 0.05, 0.075 and 0.1 μmols were prepared from the stock standard (1 mol/ml) by dissolving the salt in50% ethanol and kept at –20° C

2. 0.1 M Sodium pyrophosphate buffer ($Na_4P_2O_7.10H_2O$) pH 8.9

Dissolved 4.46 g $Na_4P_2O_7.10H_2O$ in 90 ml distilled water and adjusted to pH 8.9 with HCL. Then water was added to the final volume of 100 ml.

3. 5 mM β-NAD

Dissolved 0.41 g β-NAD in 100 ml of cold distilled water and adjusted to pH 7.0 with solid $NaHCO_3$. The solution was stored at temperature below 0°C.

4 Enzyme 3α -hydroxysteroid dehydrogenase (3α-HSD)

Prepared 330 mu/ml solution of 3α-HSD by using 0.01 M potassium phosphate buffer pH 7.2 and stored frozen.

Procedure

	Blank (ml)	Test (ml)
1. bile acid sample	-	0.1
2. 0.1 M pyrophosphate buffer	0.7	0.6
3. 5 mM β-NAD	0.5	0.5
4. 3 α-HSD	0.5	0.3
mix , incubate 25°C , 40 min, read OD 340		

Calculation

$$\text{amount of bile (mmol/l)} = \frac{E}{6.2 \times 10^3}$$

E = Absorbance of unknown sample at 340 nm.

6.2×10^3 = Molar extinction coefficient of NADH at 340 nm, 1 cm light path.

Reference:

1. Turnberg LA, Mote AA. The quantitative determination of bile salt in bile using thin layer chromatography and 3α-hydroxysteroid dehydrogenase. Clin Chim Acta 1969; 24: 253-259.

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