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Effect of age and diet composition on cholesterol metabolism in plasma leucocytes and lipoproteins in men

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INTRODUCTION

Coronary heart disease (CHD) is the leading cause of death in this country. It afflicts more than 29 million people and is responsible for about 1 million deaths per year. The incidence increases with age, from 2-3% at 15-24 years of age, to 44% at 65 years or older [1]. The process and risk factors that produce the end result of CHD can be divided into three stages, are schematically represented in Figure 1, by Connor and Connor [2]. Stage 1 in the development of CHD is the phase of relative hypercholesterolemia which occurs when a person is presented with a load of cholesterol in excess of its needs and its capacity to metabolize; and for most it occurs when the plasma cholesterol exceeds 180 mg/dl. The intensity of hypercholesterolemia is proportional to the degree that the plasma cholesterol exceeds this baseline value and the subsequent development of atherosclerosis is likewise related to the extent of hypercholesterolemia. Diet has a role in stage 1 in which it operates through its effect on plasma lipids. Although dietary factors are not crucial in the causation of hypercholesterolemia, for most Americans they are essential. After hypercholesterolemia has been present for a considerable time, stage 2 in CHD becomes manifest as atherosclerosis. The mature atherosclerotic lesion is characterized by accumulation of cholesterol esters (CE), triglycerides (TG) and phospholipids (PL) and proliferation of fibrous, calcified tissue in the arterial intima. These lesions are followed by the actual occurrence of CHD in stage 3. Risk factors: age, hypertension, cigarette smoking, stress, physical inactivity are of secondary importance and they are chiefly

operative only when hypercholesterolemia is present.

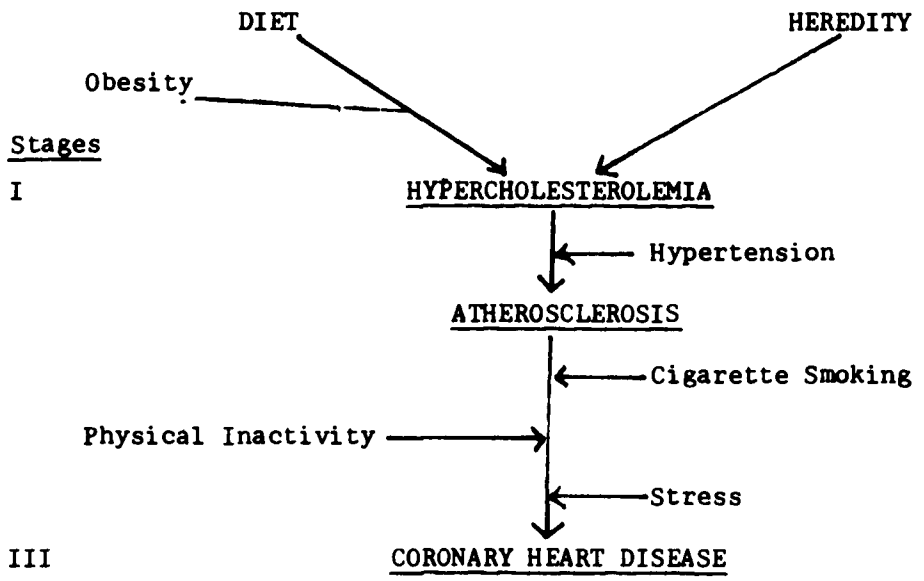

Figure 1. The stages and important factors in the development of coronary heart disease (Connor and Connor [2])

Nobody doubts that lipids are a major component of atherosclerotic lesions and the origin of these lipids may be twofold - local biosynthesis and derivation from blood. Even the lipoproteins (LP) to which the lipids are linked for transport have been blamed as having a role in CHD [3]. These complexes of protein with lipids are constructed with the polar molecules on the surface (protein and phospholipids and free cholesterol (FC)) and nonpolar molecules on the inside (triglycerides and cholesterol ester). The proteins on the surface of these LP carry the information that specifies the tissue to which different classes of lipid are to be delivered. There are four main types of LP, classified according to their size and density. These are the chylomicrons (CM),

which carry cholesterol from gut to the liver; very low density lipoproteins (VLDL) which carry cholesterol from liver to peripheral tissues; low density lipoproteins (LDL), which carry cholesterol whether obtained from diet or synthesized in the body to peripheral tissues and high density lipoproteins (HDL) which carry cholesterol not used by peripheral tissues back to liver where it may be excreted into intestine, or converted to bile acids or recycled back to peripheral tissues after incorporation into VLDL and LDL.

The relationship of VLDL and its high TG content to CHD is not clear, since epidemiological studies do not present a clear picture. The Framingham study [3], Gothenburg study [4] and the Evans county prospective study [5] found that TG and VLD were not important risk factors for CHD; while in the Tromso heart study [6] and in the Stockholm prospective study [7], TG and VLDL were recognized as important risk factors.

The causative role of increased plasma LDL in CHD has been well documented in almost all epidemiological studies. Attention has been focused on LDL as playing a key role in both the development of atherosclerotic lesions and in the regulation of cholesterol metabolism in cells other than liver cells. Brown and Goldstein [8] have developed extensive evidence that human cells have specific receptors for LDL binding. Following binding of LDL to receptors, the cell membrane invaginates to form vesicles to accommodate LDL inside the cell. These vesicles merge with lysosomes, whose enzymes digest the protein component of LDL and split off the CE with release of FC. The liberated FC has three important regulatory roles - it reduces cholesterol synthesis by suppressing the key regulatory enzymes, it activates another enzyme

which catalyzes the formation of new CE for storage, and it suppresses the synthesis of new LDL receptors, thus preventing accumulation of too much cholesterol by the cell. Lack of functional receptors could contribute to the increase in LDL concentration in two ways: peripheral cells would be unable to take up and metabolize LDL adequately. Also, the enzyme regulating cholesterol synthesis will not be suppressed and the cells would continue to produce the sterol in spite of high plasma cholesterol concentrations.

Interest in HDL was stimulated largely by the finding that HDL is inversely related to CHD. Glomset [9] hypothesized that HDL transports cholesterol from the peripheral tissues to liver, thereby reducing the amount stored there and diminishing the likelihood of its ending up in atherosclerotic lesions. Although both in vitro and in vivo evidence suggests the occurrence of such reversible cholesterol transport, it remains to be established that this mechamism diminishes atherogenesis.

Connor [10] summarized the relative pathogenicity of plasma lipids and LP for the development of atherosclerosis and this is shown in Table 1.

Table 1. The pathogenicity of plasma lipids and lipoproteins for the development of atherosclerosis [10]

Plasma Lipids and Lipoproteins	Relative Pathogenicity[a]
Cholesterol	4+
Triglyceride	1+
Chylomicrons	0
VLDL	2+
IDL	4+
LDL	4+
HDL[b]	0

[a]Graded on a scale from 0 to 4+.

[b]HDL may be protective and in itself may tend to inhibit atherogenesis.

The concept of any disease arising from a single cause is obsolete and misleading. If one accepts the view that plasma cholesterol and lipoproteins constitute the etiologic agent in atherosclerosis, there still are constellations of interacting factors which may influence not only the concentration of serum lipoproteins, but also the susceptibility of arterial intima to lipid deposition. Any modification in the chemical composition of lipoproteins (FC:CE; C:PL; C:TG and others) can have a profound effect on exchangeability, accessibility and the chemical reactivity of lipid constituents and may lead to transfusion of lipoproteins with other molecules and membranes.

The extent and severity of atherosclerosis are believed to be related to the factors including age, diet, blood cholesterol, high blood pressure, stress, physical inactivity. Therefore, this study was designed to analyze the effects of age and diet composition on cholesterol metabolism in leucocytes and plasma lipoproteins. This investigation will help understand how cholesterol metabolism changes in peripheral tissues with age, and the role of diet composition.

This dissertation consists of three papers. The first two papers deal with the development of methods and a model for studying cholesterol catabolism or bile acid synthesis in peripheral tissues. This includes the demonstration of cholesterol 7α-hydroxylase activity and presence of bile acids in human leucocytes and rat peripheral tissues. In the third paper, the leucocyte model developed in the first two papers is used for investigating the effect of age and diet composition on cholesterol metabolism in men.

REVIEW OF LITERATURE

The focus of the following review of literature is on the use of the leucocyte as biopsy tissue for studying cholesterol and bile acid metabolism and the effects of age and diet composition on blood pressure and leucocytes and plasma lipoprotein lipids. Emphasis has been placed on research done with humans, and where applicable with experimental animals. In most instances, studies were limited to data on males.

Leucocyte as Biopsy Tissue

The accumulation of cholesterol esters in atherosclerotic lesions and their lethal complications stresses the importance of understanding the regulation of cholesterol metabolism in peripheral tissues in vivo. The present techniques used for studying in vivo regulation of cholesterol metabolism in humans include sterol balance, turnover measurements after injection of isotopically labeled cholesterol or its precursors and assay of biopsy tissues [29]. These methods require steady state metabolic conditions and are time consuming and expensive or are potentially hazardous. These limitations also preclude sampling of large populations or repetitive sampling of individuals. Safe, quick and inexpensive techniques for studying the regulation of human cholesterol synthesis in vivo are needed. The use of leucocytes for studying cholesterol metabolism in peripheral tissue is under investigation.

Peripheral blood leucocytes often mirror inborn errors of metabolism in that these cells may carry the enzymatic defect that underlies the disease. This characteristic has been dramatically demonstrated in the lipid storage diseases [12] and in one form of hyperlipoproteinemia

[13]. Hsia [14] has reported that in more than 30 diseases, the leucocyte proved to be a useful indicator of an inborn error of metabolism. There is also strong evidence that in both lower animals and humans the tissue macrophages are derived from blood monocytes [15,16,17]. Since the tissue macrophages participate in the formation of both tendon xanthomas and the atherosclerotic reaction [18], it seems reasonable to believe that the human leucocyte may serve as an excellent cell model in the study of atherosclerosis. Friedman et al. [19] have reported the use of leucocyte count as a predictor of myocardial infarction and their data suggest that the predictive power of leucocyte count is similar to that of serum cholesterol or blood pressure.

Ziats and Roberston [20] have also reported that blood monocytes may play a key role in arterial smooth muscle cell proliferation, a recognized event in the initial stages of atherosclerosis. All types of leucocytes contain the full range of lipid species encountered in most mammalian tissues. Lipids constitute about 5% of the wet weight of normal human leucocytes, and about one-third of the total lipid is neutral lipids, primarily free cholesterol and triglycerides. Gottfried [21] and Jaffe and Gottfried [22] have reported 5.33 μg cholesterol per 10^6 cells in granulocytes and 3.24 μg per 10^6 cells in lymphocytes. They also observed very small amounts of cholesterol esters (CE) in human lymphocytes and granulocytes. Suzuki [23] also reported a predominance of free cholesterol (FC) in rabbit leucocytes (FC:CE=3:1) in contrast with predominance of cholesterol esters in plasma (FC:CE=1:2.5). Berghoff and Glatzel [24] suggested that leucocytes may have a role in the transport of absorbed fats when they observed a 20% increase in lipid incorporation in

leucocytes after a high fat meal.

Mixed human leucocytes [25] and isolated lymphocytes [26] can synthesize cholesterol from acetate. Fogelman et al. [27] have shown that the monocytes are five times more active than lymphocytes in cholesterol synthesis from acetate or mevalonate; granulocytes, however can not synthesize cholesterol from either precursor. Higher activity in monocytes may be attributed to their larger cell size compared to granulocytes and lymphocytes. Fogelman also speculated that a relationship might exist between the short life span of granulocytes in vivo (1-2 days) and the long life span of monocytes (3 months without division) and their respective abilities to synthesize sterols and hence replace cholesterol lost from their membrane.

In a number of other ways also, leucocytes are an important species of cells for the study of cholesterol metabolism in peripheral tissues. Leucocytes behave as a nonendocrine, extrahepatic tissue and reflect LDL regulation of peripheral tissue cholesterol metabolism [28]. Their presence in the peripheral blood means that unlike other nonhepatic cells, such as fibroblasts, they are more easily available for repeated analysis; artifacts caused by passage of other nonhepatic cells through tissue culture are avoided; and activity of rate limiting enzymes in cholesterol metabolism can be determined in freshly isolated, unincubated uncultured leucocytes which have been in contact with endogenous lipoproteins, thus, reflecting the situation in vivo. Thus leucocytes can help monitor the response of peripheral tissue to diet, drugs and surgical intervention. A person could serve as his/her own control for effects of various treatments. Lack of diurnal variation in leucocytes [29]

also facilitates experimentation since blood samples can be obtained at any convenient time of day.

Effects of Age

Leucocytes

Helman and Rubenstein [30] studied the effect of age on total leucocyte count and observed that it does not change with age. Shapleigh et al. [31], Otani [32], and Zacharski et al. [33] also observed that total leucocyte and lymphocyte count remained constant from the 3rd to the 10th decade.

Mackinney [34] analyzed blood counts of 184 inpatients and 884 outpatients. They observed that the absolute lymphocyte count decreased from 2023 in people aged 20-29 years to 1872 in a 60-69 year age group. The lymphocyte count remained constant for ages 20-45 years then declined in subsequent years. This decrease may partially explain the decrease in cellular and humoral immunity observed in older people. They observed that granulocyte count does not show age dependent variations and remains constant throughout life. Reddy and Goh [35] also reported a decrease in lymphocyte count from 2548 in 20-40 year individuals to 2314 in 60-69 year people.

Letzky and Papisowa [36] studied the total lipid content of blood leucocytes in various age groups in men. They reported an increase in the lipid content of neutrophils and monocytes from birth to 35 years of age, after which it remained constant.

Blood pressure

It is unanimously agreed at the present time that systolic and diastolic blood pressure are risk factors of CHD [37,38,39]. In the Evans county study [40], the CHD prevalence was 2.3 times higher in the subjects whose blood pressure exceeded 160/90 mm Hg than in those with lower values. The western collaborative study [41] showed the CHD incidence to be 3.6 times higher in the subjects with a diastolic blood pressure over 94 mm Hg than in those with a diastolic blood pressure below that value. A study based on the autopsy materials of the International Atherosclerosis Project [42] revealed a significant and positive correlation between hypertension and the severity of atherosclerosis. However, Chapman and Massey [43] noted no correlation between CHD and elevated blood pressure in their study of men aged below 60 years.

Shaw et al. [44] reported that systolic blood pressure shows little change from 25 to 65 years of age, but after the age of 65, it increases markedly. Diastolic blood pressure significantly increases from 25 to 35 years of age with little further increase as aging progresses. Juustila [45] observed that in men systolic blood pressure increases steadily with age from 40 to 59 years, while diastolic pressure remains unchanged.

The results of the first health and nutrition examination survey (HANES) in the U.S. [46] show that the mean systolic blood pressure in white men increased significantly and consistently with age from 119 mm Hg at age 18-24 years to 149 mm Hg at ages 65-74 years. The increment was lowest among young men 18-24 years (averaging 0.1 mm Hg per year); it then increased at a fairly steady rate from 35 years and older (an average of 0.8 mm Hg per year). The mean diastolic pressure in white

men also increased with age, reaching a maximum at ages 45-54 years, at a rate which declined from an average of 0.5 mm Hg per year in age 18-34 years to 0.3 mm Hg per year at 35-54 years. The increase in diastolic pressure with age occurred at a slower rate and was less consistent than that of systolic pressure. The increase in mean diastolic pressure was from 73.8 mm Hg in age 18-24 years to 86.3 mm Hg in age 55-64 years. The proportion of individuals with both systolic and diastolic blood pressure substantially elevated (systolic pressure of at least 140 mm Hg and diastolic of at least 90 mm Hg) increased markedly with age from 4% in ages 18-24 years to 36% in ages 65-74 years.

A positive association of serum cholesterol with blood pressure, though often suggested, has not been clearly demonstrated in man. It has, however, been shown by Deming et al. [47] that lowering blood pressure of hypertensives with a variety of agents does result in a fall in their serum cholesterol. Blood pressure does have some effects on cholesterol metabolism. Adel et al. [48] showed that in vivo hypertensive rats synthesize cholesterol more rapidly than do normotensive rats. The changes ranged from 10 to 100 fold increase. They suggested that this effect on cholesterol metabolism is not a direct result of blood pressure, but reflects a change in tissue brought about by pressure. Juustila [45] observed that systolic hypertension was accompanied by high serum cholesterol. On the other hand, Olsson et al. [49] found that serum cholesterol had no correlation with either systolic or diastolic pressure. William et al. [50] did not observe any association between high density lipoprotein and blood pressure, but Hulleys and Ashman [51] observed that high HDL is associated with an increase in diastolic pressure.

Cholesterol levels in plasma and lipoproteins

A rise in mean serum cholesterol with age has been reported in many studies. The mean serum cholesterol level in men tends to rise until a plateau is reached between the ages of 40-65 years, followed by a decline. Reported values at the time of peak vary from 230 to 245 mg/dl plasma [52,53,54] and in some instances higher levels have been noted [55,56]. Adlersberg et al. [57] observed that the increase in cholesterol with age occurs at the rate of 3.6 mg per dl of plasma per year. Boberg et al. [64] observed a slight continuous increase in plasma cholesterol from 25 to 45 years of age, with little change from middle to old age in men. The HANES report [65] showed an increase in mean serum cholesterol with age from 189 mg/dl in 18 to 24 year old men to 251 mg/dl in 65 to 74 year old men, which is an increase of 32%. The increase was steady from the 18-24 to 35-44 year age groups followed by smaller increments which reached a maximum at age 55 to 64 years and then slightly declined for the oldest age group studied (65-74 years). Serum cholesterol levels of men at ages 45-54 years showed a plateau following the early increase. The proportion of men with serum cholesterol exceeding concentrations 260 mg/dl varied from a low of 4% in 18-24 year old men to 26% in 45-54 year olds.

Lewis and coworkers [58] observed that LDL is more sensitive than serum cholesterol as a risk factor of CHD. They also reported that the highest LDL values are seen at ages 40-49 years. Goffman and Tandy [59] reported that LDL are strongly related to the risk of CHD only early in life (up through the 4th decade), with the predictive relationship weakening steeply in the next two decades; by 50-55 years of age there is

little if any relationship of any serum lipid parameter with risk of future evolution of CHD. They also observed an increase in LDL with increase in chronological age in men during the 3rd and 4th decade, with peak LDL levels being reached at approximately 45 years of age, followed by a decline. The VLDL in men showed an upward trend during the 3rd decade, with an even steeper upward rise in the 4th decade, peak levels being reached during the 5th decade followed by declining levels as age increased. Low positive correlations in LDL and VLDL with increase in systolic pressure were also observed.

Fredrickson et al. [60] have also observed an increase in plasma cholesterol, VLDL and LDL with age. They noted that the increase in LDL is slow but continuous through the 3rd decade, after which the rise takes place is at a more perceptible rate. They explained that this pattern could be attributed to cessation of growth and also to sendentary habits after age 30. Clark et al. [61] did a 12-year longitudinal study of serum lipids on the subjects from the U.S. Military Academy. They observed an increase in mean serum cholesterol from 183 to 237 mg/dl with increase in age from 19 to 31 years. An increase in LDL and downward trend of HDL were also noted. Results of Scott [62] and Allard and Goulet [63] also corroborate the age associated trends in LDL.

Connor et al. [66] reported that in normal subjects the increase in plasma cholesterol is entirely in LDL, with insignificant increments in HDL and VLDL. Olsson and Carlson [67] also reported a significant positive correlation of serum cholesterol with VLDL (0.57) and LDL (0.81).

Males show stable HDL concentrations during the first decade of life, lower values during puberty and adolescence, followed by stable values up

to 50-55 years, an increase around 60 years and a plateau thereafter [68]. Most population studies have reported no association between plasma and HDL cholesterol, while a weak positive association has been described by others [69]. Examination of the relationship between HDL and LDL cholesterol had led to reports of either no significant correlation [70] or a negative correlation [71].

The proportion of free to cholesterol esters (FC:CE) in the plasma is generally considered to be relatively constant. As early as 1936, Sperry [72] stated that the proportion of free to esterified cholesterol remains quite stable despite large differences in the total amount of cholesterol present. Similar observations have been made by Boyd [73] and Foldes and Murphy [74]. However, Leonard et al. [75] reported that as the total amount of cholesterol in plasma increases, the increases in the free cholesterol fraction becomes proportionately greater than the increase in the esterified fraction. Lopez et al. [76] also observed that with advancing age there is a trend towards a proportional increase in the free cholesterol, but this was not statistically significant.

No studies have been noted reporting the effects of age on the proportion of free and esterified cholesterol in lipoproteins. The results of Bragdon et al. [77], Oncley et al. [78], Oncley [79] and Shen et al. [80] show the following FC to CE ratio in different lipoproteins.

Lipoproteins	FC:CE ratio (range)
VLDL	1:1.2 to 1:1.6
LDL	1:2.5 to 1:4.4
HDL	1:1.6 to 1:5.0

Bile acid levels in plasma lipoproteins

Uchida et al. [81] and Dupont et al. [82] observed that the bile acid pool size of male rats does not change with aging, whereas Beher et al. [83] have reported a decrease in the bile acid pool size of male rats with increase in age. They observed that both cholic and chenodeoxycholic acid pool size decreased with aging.

Bennion et al. [84] found no age effects on the bile acid pool size of normal men and women.

No studies were noted reporting the presence of bile acids in lipoproteins other than HDL. According to Middlehoff et al. [85] the bile acid content of HDL in normal people is 8.5 nmol/ml plasma.

Cholesterol and bile acid synthesis

Most of the cells of the body have the capacity to synthesize cholesterol, but physiologically the liver and the intestine account for over 90% of the body's total synthesis. The synthetic pathway of cholesterol can be divided into four major steps. The first step is the production of 3-hydroxy-3-methylglutaryl CoA(HMGCoA) by condensation of three molecules of acetyl CoA by cystolic acetoacetyl CoA thiolase and HMGCoA synthase. The next step involves reduction of HMGCoA to mevalonic acid and is catalyzed by microsomal enzyme HMGCoA reductase and represents the overall rate limiting step of cholesterol synthesis[86]. Under physiological conditions, the HMGCoA reductase activity correlates with sterol production. Conversion of mevalonic acid to squalene with loss of one carbon at pyrophosphomevalonate decarboxylase comprises the third step. In the fourth step, cyclization of squalene with

the loss of 3 methyl groups yields cholesterol. Andersen and Dietschy [86] showed that serum lipoproteins exert 3 different types of effect in regulating the cholesterol synthesis in the body. These include regulation of chclesterogenesis in liver by CM remnants, in extrahepatic, nonendocrine tissues by LDL, and in endocrine tissues by HDL.

Decrease in hepatic synthesis with aging has been reported by Bloch et al. [87], Srere et al. [88], Trout et al. [89], and Yamamoto and Yamamura [90]. Kritchevsky et al. [91] observed that the decrease in cholesterol synthesis with age occurs not only in liver, but also in extrahepatic tissues of the rat: spleen, kidney and colon. However, Story et al. [92], and Dupont et al. [82] did not observe any significant effect of age on cholesterol synthesis in rat liver.

The ultimate degradation of cholesterol involves breakage of the ring structure, the capability of which mammalian tissues do not possess. The transformation of cholesterol can only take place in those tissues which synthesize bile acids or steroid hormones. The first specific reaction in the biliary acid metabolic pathway is hydroxylation of cholesterol on the 7α position [92]. This reaction is catalyzed by microsomal enzyme cholesterol 7α hydroxylase and is the rate limiting step of the entire pathway [93]. Hruza and Wachtlova [94], Yamamoto and Yamamura [90], and Story and Kritchevsky [95] observed that bile acid synthesis in rat liver decreased with aging. Dupont et al. [96] also observed a decrease in rate of turnover of cholesterol to bile acids and proposed that decreased cholesterol catabolism can lead to an increase in serum cholesterol with aging.

Plasma and lipoprotein phospholipids

A significant increase in plasma phospholipid (PL) concentration with age has been reported in many studies [97-101]. Boberg et al. [64] observed that increase in plasma PL is slight but continuous from 25 to 45 years and after middle age there is very little change in PL levels. On the other hand, Clark et al. [61] and Berlin et al. [102] found that age had no effect on plasma PL levels, they remained more or less constant throughout life.

Phospholipids are believed to be protective against a high cholesterol level, so that the cholesterol:phospholipid (C:PL) ratio has been advocated as an index of atherogenesis. A high incidence of atherosclerosis is found in patients with nephrosis, hypothyroidism and essential xanthomatosis, where the plasma cholesterol exceeds that of phospholipids. Atherosclerosis is not found in biliary obstruction or Von Gierk's disease where plasma phospholipids exceed cholesterol, even though the latter is markedly above normal [103]. In normal individuals the ratio of C:PL tends to remain constant even though there are wide fluctuations in the normal level of cholesterol and phospholipid [104]. Aldersberg et al. [57] reported that C:PL ratio increases with age and for each 1 mg/dl increase in plasma cholesterol, the increase in phospholipid is 0.67 mg/dl. However, Clark et al. [61] found that C:PL ratio remained constant during their 12-year longitudinal study of serum lipids.

Plasma and lipoprotein triglycerides

Pelkonnen et al. [105] and Vik et al. [6] in the Tromso heart study and Carlson et al. [7] in the Stockholm prospective study found

elevated plasma TG to be a significant risk factor for CHD, whereas in the Gothenburg study by Wilhelmsen [4] and the Evans country prospective study [5] TG were not a risk factor for men.

Carlson [97] reported that serum triglycerides (TG) increased significantly with age in men from 0.85 mg/dl at age 26-30 years to 115 mg/dl at age 40, and then remained constant to age 73 years. Fredrickson et al. [60] observed an increase in plasma TG from 61 mg in 0-17 year old individuals to 104 mg/dl by 50-59 year old people. Carlson and Bottiger [106] in the same year reported an increase in plasma TG in men from 106 mg/dl in 20-24 age group to 151 mg/dl in 45-49 age group and a decrease to 139 mg/dl in 60-64 year old men. Zweers et al. [107] also corroborated this age associated increase in plasma TG.

Olsson and Carlson [67] studied serum lipoproteins of a random sample of 40-year-old men and reported significant positive correlations between serum TG and all lipoproteins TG, the strongest being with VLDL TG (0.95). They concluded that the increase in serum TG reflects mainly VLDL TG. Tobey et al. [108] reported that the age dependent increase in plasma TG is due to an increase in VLDL-TG secretion and a relative decline in efficiency of VLDL-TG removal from plasma.

The use of cholesterol:triglyceride ratio to predict atherosclerosis has been proposed. An increase in C:TG ratio in VLDL may signify accumulation of atherogenic cholesterol-rich remnants of VLDL catabolism [109]. An increase in LDL TG relative to C could reflect a more liquid crystalline, neutral lipid core [110] which is in turn associated with decreased atherogenicity in the primate model [111]. Knopp et al. [112] examined a random sample of Caucasian men and women and observed no

significant effect of age on plasma lipoprotein C:TG ratio. The mean C:TG ratio for VLDL in men was 0.34, for LDL 7.84 and for HDL 4.50. They concluded that atherosclerosis of aging is not associated with a change in C:TG in lipoproteins.

Food intake

Munro [113] observed that the mean food energy calorie and protein intakes were decreased with increasing age, the decrease being from 3060 Kcal and 91 gm of protein in 22-year-old men to 2440 Kcal and 83 gm of protein in 60 year old men. The percentage of calories from protein increased from 11.9% in 22 year old men to 13.6% in 60 year old men. In the HANES report [114], the peak calorie and protein intakes in white males were in the 18-19 year old group and thereafter they decreased, with the reduction being from 2973 Kcal and 114 gm protein in 20-24 year old men to 2112 Kcal and 74 gm protein in 55-64 year old men. In the survey the mean calorie intake of 45-74 year old men met, on an average, about 80% of the recommended allowance [115].

McGandy et al. [116] reported a significant age related decrease in total calorie intake in men; the annual average decrease was 12.4 Kcal/day. The percentage of calories derived from protein remained constant with increasing age, whereas calories from fat dropped from 42% in the 45-54 year old group to 36% in the 80 year old group; calories from carbohydrate increased. The daily intake of fat decreased with age from 123 gm in 20-34 year old men to 99 gm in 65-74 year old group, and there was a concomitant decrease in cholesterol intake. Various reports have noted that the mean serum cholesterol intake decreases in the male population over

ages 50-60 years and this age associated decrease could be accounted for by a decreased fat intake.

Effect of Diet Composition on Plasma Lipids

No factor has been more highly correlated with serum cholesterol and lipoprotein levels than diet, whether by epidemiologic or metabolic studies. Delangen [117], Snapper [118], Dock [119] and Keys [120] were among those who called attention to the strong positive associations of dietary cholesterol, total dietary fat and animal foods to the occurrence of coronary heart disease (CHD). Product moment correlation coefficients computed between human CHD mortality rates and average daily intake of various nutrients for 30 countries are shown in Table 2. Dietary factors which correlate positively and significantly with the mortality rate from CHD include dietary cholesterol, animal protein, animal fat, total saturated fat, meats and eggs, as well as total calories and sugar.

Carbohydrates

High carbohydrate diets, at least on a short term basis, induce elevated levels of TG and VLDL and only a slight increase, if any, on plasma total cholesterol level [121, 122, 123]. Populations, however, consuming high carbohydrate diets habitually have a low incidence of atherosclerosis and do not have markedly elevated plasma TG [124].

In the 1960s it was suggested by Yudkin and Roddy [125] that the high sucrose consumption of the European American population might have an important role in the pathogenesis of CHD. Anderson et al. [126], Grande et al. [127] and Keys [128] have shown that in isocaloric feedings, carbohydrate derived from sucrose and lactose elevated, to some extent,

Table 2. Correlations between the mortality rates from coronary heart disease in men aged 55-59 years and the intake of certain foods and nutrients[a] (Delangen [117]; Connor and Connor [2])

Positive correlations ($p < 0.05$)	
Animal protein	0.782
Cholesterol	0.762
Meat	0.697
Total fat[b]	0.676
Eggs	0.666
Sugar	0.638
Total calories	0.633
Animal fat[b]	0.632
No correlations ($p < 0.05$)	
Plant sterols	0.144
Fish	0.013
Vegetable fat[b]	0.011
Vegetables	0.009
Negative correlations ($p < 0.05$)	
Starch	-0.464
Vegetable protein	-0.403

[a]Data derived from the national statistics on food consumption and deaths from 30 different countries.

[b]Data available from only 29 countries for these nutrients.

the serum cholesterol levels while those derived from cereals, grains, fruits and vegetables were without that effect. Little et al. [129] did not observe an increase in serum lipids by sucrose when compared to starch. Hyperlipidemic effects of dietary sucrose in humans were also not verified by other investigators [130,131].

Protein

The possible effects of the dietary protein (both single protein sources and mixtures of several different proteins) on human plasma lipid levels has been extensively studied. Connor et al. [132] showed that the proteins consumed in mixed human diets have no significant effects upon plasma lipid levels. Anderson et al. [133] also did not observe any significant changes in plasma lipids with different quantities or sources of protein intake, so long as protein and amino acid requirements were met. Truswell [134] observed that in severe protein deficiency, plasma cholesterol in LDL and VLDL is lowered, but HDL is not affected.

Cholesterol

Reports on the effect of dietary cholesterol on plasma cholesterol have been very confusing. Keys et al. [135] and Ahrens et al. [136] reflected the prevalent opinion of the 1950s when they stated that the serum cholesterol levels are independent of dietary cholesterol. Wilson et al. [137], Grundy [138], and Mahley [139] also observed that the human response to dietary cholesterol is limited. They pointed out that most animal species are more sensitive to dietary cholesterol than is man and that humans have a remarkable ability to resist the absorption of dietary cholesterol and that hepatic cholesterol synthesis is suppressed in the

dietary cholesterol in amounts above 110 mg and up to 600 mg per day increased serum cholesterol concentrations. Beyond 300 mg/day, the rate of increase was small with increasing cholesterol intake, an observation suggesting a plateau effect. He also concluded that dietary cholesterol increased the body pool of cholesterol (both plasma and tissue) as well as the bile acid excretion, but did not suppress biosynthesis of cholesterol.

The effect of dietary cholesterol on plasma cholesterol is probably not linear, and based on a review of experimental data in the literature, Keys et al. [141] estimated that one egg per day (250-325 mg cholesterol) added to the 600-700 mg of cholesterol consumed by the average American male would increase his dietary cholesterol by nearly 50% but his serum cholesterol concentration would increase by only 5 to 6 mg/dl. They did not estimate what effect a reduction in cholesterol intake by an amount similar to the addition of an egg would have. Previous reports of Keys et al. [142] suggest that a dietary cholesterol reduction, would lower serum cholesterol by an amount greater than concentrations resulting from an addition of an egg.

Fat

The amount and kind of fat in the diet have a considerable effect upon the plasma lipid levels. A diet rich in polyunsaturated fats is generally associated with lower plasma lipid levels than one containing larger quantities of saturated fats [143]. As early as 1952, Kinsell et al. [144] reported that animal fats raised plasma cholesterol, while vegetable fats lowered it. Ahrens et al. [136] have observed a positive correlation between the iodine value of dietary fats and their cholesterol

lowering properties. Keys et al. [145] reported that saturated fatty acids with more than 12 but less than the 18 carbon atoms raised plasma cholesterol concentration, while polyunsaturated fatty acids had an opposite, but weaker effect than that of saturated fat. They observed that 2 gm of polyunsaturated fat were required to counteract the effect of 1 gm of saturated fat on serum cholesterol level.

Jollife [146] proposed that the effect of dietary fats on the serum cholesterol was a function of their ratio of polyunsaturated to saturated fatty acids (P/S). This concept that saturated fatty acids or dietary fats with low P/S are hypercholesterolemic is widely accepted. Ericcson et al. [147] compared cholesterol-free formula diets of different P/S ratio of 0.1, 0.7, 1.5 and 1.6. They found that a high P/S ratio 1.6 or an extremely low ratio in the diet resulted in an identical plasma cholesterol levels and concluded that with a cholesterol free formula diet the plasma cholesterol level is unaffected by the variation in the P/S ratio of diet. Shepherd et al. [148] studied the effects of diets rich and poor in polyunsaturated fats, with a P/S ratio of 0.25 and 4.0, and providing 400 mg of cholesterol per day. They found that when compared to the diet with low P/S ratio, polyunsaturated fat diet decreased plasma total and LDL cholesterol and also HDL cholesterol by 33%.

Populations having habitual diets rich in carbohydrate and low in fat (and usually low in cholesterol and saturated fats) have not only lower LDL cholesterol levels than westernized urbanized populations, but also lower HDL cholesterol levels [149].

Considerable controversy still exists concerning the possible mechanisms by which dietary saturated and polyunsaturated fats influence

the plasma cholesterol levels. In normal humans, given polyunsaturated fat diets, an increase in the fecal output of bile acids and neutral sterols has been reported [150]. Spritz et al. [151] and Kritchevsky et al. [152], however, failed to observe increased bile acid excretion or synthesis with polyunsaturated fat diets. They suggested that redistribution of cholesterol body pools is responsible for the cholesterol lowering effect of polyunsaturated fats. Spritz and Mishkel [153] have proposed that changes in lipoprotein structure by polyunsaturated fats cause cholesterol to leave plasma and enter tissues. The mechanism of action involved remains unsettled.

Total calorie intake

The excessive calories derived from any source whether fat, carbohydrate, protein and/or alcohol, induce hypertriglyceridemia in some individuals by providing increased substrate for TG synthesis in the liver. This condition is usually associated with hypercholesterolemia which is brought about partly by the concomitant increased intake of dietary cholesterol and fat and by the VLDL which transport both the increased TG content and cholesterol. According to Anderson and Keys [154] who used diets with the traditional American (U.S.A.) fat distribution, each 1% rise in calorie intake above the amount needed to maintain body weight will raise the serum cholesterol by about 2 mg/dl. But this increasing serum cholesterol reaches a plateau after approximately 10 weeks, regardless of the positive caloric balance and weight gain that continue beyond 10 weeks.

PAPER I. CHOLANOIC ACIDS AND CHOLESTEROL 7α-HYDROXYLASE ACTIVITY IN HUMAN LEUCOCYTES

Cholanoic acids and cholesterol 7α-hydroxylase activity in human leucocytes

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ABSTRACT

Evidence is presented for the identification of cholanoic acids and cholesterol 7α-hydroxylase activity in human leucocytes. Mononucleated cells contained most of the detectable cholanoic acids. Cholesterol 7α-hydroxylase activity was present only in the mononuclear cells. These data suggest that cholanoic acids in leucocytes may have originated by local biosynthesis. Because of their lipid solubilizing properties, the cholanoic acids might have a function in the phagocytic activity of leucocytes.

INTRODUCTION

The conversion of cholesterol into bile acids is catalyzed by microsomal enzymes and requires the transformation of cholesterol into 7α-hydroxycholesterol. Cholesterol 7α-hydroxylase, the enzyme that mediates this transformation, controls the overall rate of bile acid synthesis [93]. Despite evidence that bile acids are present in tissues other than liver [155,156], it still is generally thought that this enzyme is localized only in liver microsomes [157,158]. Herein, we report both the presence of cholanoic acids and cholesterol 7α-hydroxylase activity in human leucocytes and verification that 7α-hydrocholesterol is the end product of the cholesterol 7α-hydroxylase assay.

MATERIALS AND METHODS

Venous blood samples from normal healthy subjects were obtained by venipuncture in vacutainer tubes containing heparin. Mononuclear cells were isolated by layering over Ficoll-Paque (Pharmacia Fine Chemicals)[159]. Monocytes were collected by adherence to culture flasks and nonadhering lymphocytes were eluted. Granulocytes were isolated from the lower Ficoll-Paque band. Leucocyte microsomes were prepared by following the method of Young and Rodwell [29]. Microsomal protein was determined by the Lowry procedure [160].

Cholanoic acids from lyophilized monocytes, lymphocytes, and granulocytes were extracted with chloroform:methanol (1:1). They were separated from other acidic lipids by thin-layer chromatography in a solvent system of toluene: ethanol:methanol:water:ammonium hydroxide (50:20:14:3:1)[161]. Reverse phase high pressure liquid chromatography (HPLC), coupled with an ultraviolet detector set at 193 nm, was used for identification and quantitation of conjugated bile acids [162]. Samples prepared in the elution solvent mixture (2 propanol: 8.8 mM potassium phosphate buffer, pH 2.5, 160:340), were analyzed on a 44 mm x 30 cm μ Bondapak/C-18 column (Waters Associates) by eluting at 1 ml/min. Quantitation was done by comparing the sample peak area with corresponding standards.

Cholesterol 7α-hydroxylase assays were done by using the Carlson et al. method [163] with the following modifications. A 0.3-ml sample of microsomal suspension in homogenizing buffer [29] was incubated at 0°C for 10 minutes with 0.3 ml of potassium phosphate buffer (pH 7.4) containing 35 mM cysteamine, 0.15 ml of 4.5 mM $MgCl_2$, and 0.15 ml of

cholesterol suspension [0.1 μCi of (4-^{14}C) cholesterol (54 μCi/mmol) solubilized with the aid of 0.36 mg of Tween 80]. This allowed equilibration of exogenous and microsomal cholesterol. A substrate cofactor solution (0.15 ml) containing 0.75 mM NADP, 3 mM glucose 6 phosphate, and 0.15 units of glucose 6 phosphate dehydrogenase was then added, and the mixture was incubated for 40 minutes, at 37°C with shaking. Blanks containing boiled microsomes were run also. The reaction was stopped with 2 ml of ethanol. The assay conditions were such that, during incubation, 7α-hydroxycholesterol formed from cholesterol was not converted into intermediates further along the pathway or to secondary metabolites.

Confirmation of 7α-hydroxycholesterol as the end product of this assay was accomplished by using electron-impact mass spectrometry. A Finnigan GC/MS/DS model 2709 was used to obtain the spectra by using the following conditions: 25 eV ionizing voltage, ion source temperature 260°C, and 8.0 Kv acceleration voltage. Samples were introduced into the source chamber with a solid sampling probe. Commercially obtained 7α-hydroxycholesterol (Steraloids, Inc.) was used as a reference standard.

RESULTS AND DISCUSSION

The quantitation by HPLC of the cholanoic acids found is shown in Table 1. All cell types contained cholanoic acids. The granulocytes had very little as compared with concentrations in monocytes and lymphocytes (the mononuclear leucocytes). The cholesterol 7α-hydroxylase activity in different leucocytes (Table 2) is expressed as the percentage of ^{14}C-cholesterol added to incubation medium that is converted to ^{14}C 7α-hydroxycholesterol, because the amount of endogenous cholesterol participating in the reaction was not determined. Most of the activity is in mononuclear leucocytes, monocytes being 2.5 times as active as lymphocytes. This finding is consistent with the observation made by Fogelman et al. [27] that monocytes are 5 times more active than lymphocytes in cholesterol biosynthesis. We could not detect any activity in granulocytes, and this was not unexpected because inability of granulocytes to synthesize cholesterol has been demonstrated [164].

The mass spectrum (Figure 1) of 7α-hydroxycholesterol exhibited a molecular ion at m/e 402. The fragmentation observed had predominant M-18 (80%) and M-2x 18 (33%) peaks corresponding to the loss of water molecules. The peak at 253 suggested side-chain scission between C-17 and C-20. The spectrum of the sample isolated from the leucocytes was virtually identical with that of a reference standard (Table 3).

Cholanoic acid presence and synthesis in leucocytes has not been reported before. The probability of formation of the cholanoic acids in the mononuclear leucocytes by local biosynthesis is suggested by the presence of cholesterol 7α-hydroxylase, considered to be the rate-

limiting enzyme in the biliary acid biosynthesis. Hydroxylated sterols do not accumulate in cells, so further reactions to form the bile acids identified are likely. The function and importance of these compounds in phagocytosis and immune responses will be an important sequel to these findings.

Table 1. Cholanoic acids in human leucocytes[a] (range of 4 subjects)

Leucocyte	TC	TCDC	TDC	GC	GCDC	GDC
Monocytes (μg per 10^6 cells)	5.35 to 5.65	9.46 to 13.5	ND[b]	14.15 to 20.29	8.4 to 13.83	ND
Lymphocytes (μg per 10^6 cells)	1.8 to 2.2	2.6 to 3.05	ND	4.15 to 4.21	3.04 to 3.26	0 to 2.09
Granulocytes (μg per 10^6 cells)	0.108 to 0.211	0.224 to 0.315	ND	0.219 to 0.348	0.314 to 0.421	ND

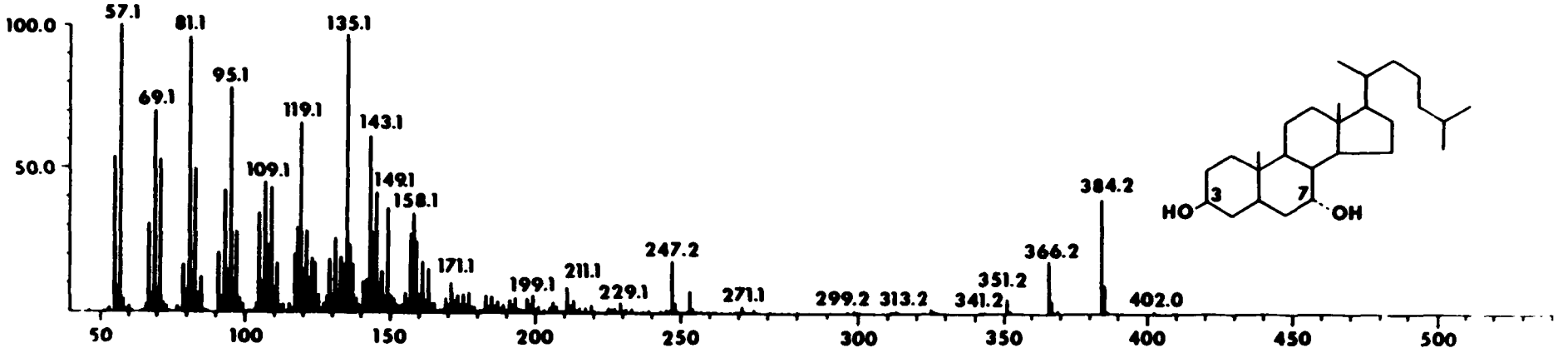
[a]TC = Taurocholic; TCDC = Taurochenodeoxycholic; TDC = Taurodeoxycholic; GC = glycocholic; GCDC = Glycochenodeoxycholic; GDC = glycodeoxycholic acids.

[b]Not detectable.

Table 2. Cholesterol 7α-hydroxylase activity in human leucocytes (mean ± SD of 4 subjects). Percentage conversion of cholesterol to 7α-hydroxycholesterol per 40 min per mg microsomal protein

Leucocyte	Activity
Monocytes	0.760 ± 0.252
Lymphocytes	0.301 ± 0.112
Granulocytes	Not Detectable

MASS SPECTRUM OF 7 - HYDROXYCHOLESTEROL.



MASS SPECTRUM OF 7 - HYDROXYCHOLESTEROL ISOLATED FROM HUMAN LEUCOCYTES

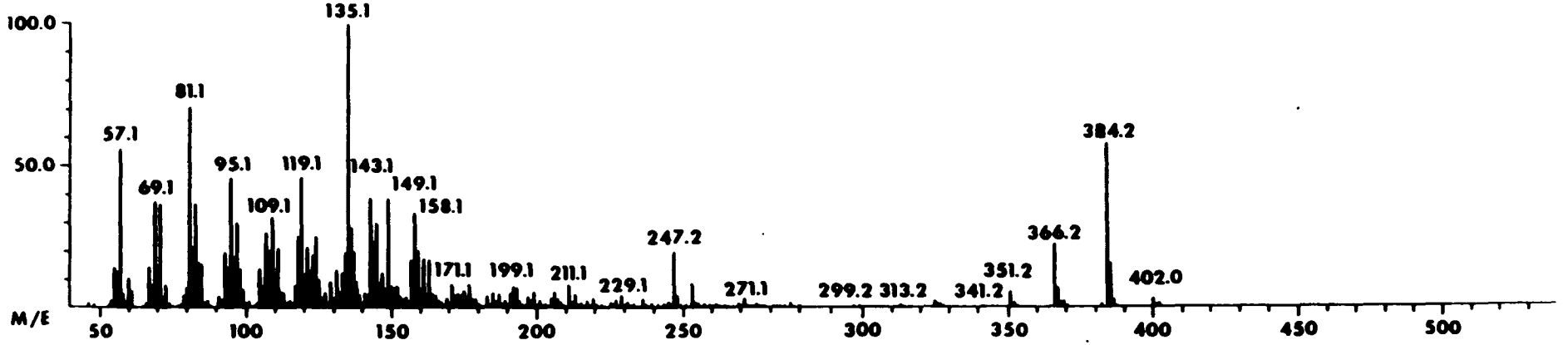


Table 3. Fragment ions and relative intensities of 7α-hydroxycholesterol isolated from human leucocytes vs. standard

	m/e	Human leucocyte	Standard
$M^{+}_{\cdot}$	402	2	1
$[M-n\cdot 18]^{+}_{\cdot}$	384	58	41
$[M-2\cdot 18]^{+}_{\cdot}$	366	23	20
$[M-2\cdot 15+15]^{+}$	351	5	2
	313	0.5	0.5
	299	0.5	0.5
	271	3	3
$[M-149]^{+}$	253	12	10
	247	15	18
	229	4	5
	211	10	11
	199	7	8
	171	9	12
	158	35	45
	149	39	43
	143	39	61
	135	100	100
	119	47	66
	109	33	44
	95	46	78
	81	71	96
	69	37	70
	57	59	100

PAPER II. CHOLANOIC ACIDS AND CHOLESTEROL 7α-HYDROXYLASE ACTIVITY IN RAT EXTRAHEPATIC TISSUES AND HUMAN LEUCOCYTES

Cholanoic acids and cholesterol 7α-hydroxylase activity in rat extrahepatic tissues and human leucocytes

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ABSTRACT

The possibility of significant concentrations of bile acids in tissues other than liver and gut has been raised for a number of years. Problems of isolation and quantitation have hampered efforts to verify that possibility. Rat tissues and human leucocytes have been used in this study to show the identity, quantity and possible origin of cholanoic acids. Thin layer chromatography (TLC) and high performance liquid chromatography (HPLC) were used for isolation of cholanoic acids and the latter for quantitation. Quantitation also was done using a 3α-hydroxysteroid dehydrogenase enzyme assay. Structures of glycocholic and glycochenodeoxycholic acids were confirmed by electron impact and chemical ionization mass spectrometry. The taurine conjugated acids isolated from tissues had spectra which matched authentic compounds.

Rat kidney, heart, lung, adipose tissue, and human leucocytes were shown to contain these compounds. In addition, cholesterol 7α-hydroxylase activity was found to be present in all the tissues examined. The unequivocal confirmation of cholanoic acids in nonhepatic tissues in significant amounts (7-20%) of hepatic concentration and the ability of tissues to synthesize hydroxylated sterols accentuate the importance of learning their functions in the tissues.

INTRODUCTION

Unequivocal physical identification of individual nonhepatic bile acids is lacking, though there is evidence of the presence of cholanoic acids in brain [165] skeletal muscle, kidney, pancreas, and adipose tissue [155]. The presence of cholanoic acids in tissues raises the question of whether the compounds originated by local biosynthesis or by transfer from blood after synthesis in the liver.

Bile acids generally occur in nature in free and conjugated forms and both are bound to proteins. Quantitative isolation of bile acids is difficult because of the physiochemical properties of bile acids and the presence of other compounds, particularly the acidic pigments and fatty acids. Numerous methods have been described for the isolation of bile acids from various sources [166-167]. Methods which utilize a vigorous hydrolysis step destroy the natural form of the bile acid and the separation of fatty acids from bile acids, particularly lithocholic and other less polar bile acids, poses a problem. Removal of fatty acids from samples is essential as they interfere with bile acid analysis by gas liquid chromatography (GLC). This is usually done by thin-layer chromatography (TLC) and different methods have been proposed for the separation of fatty acids from bile acids [168-170].

Evidence is presented herein for identification of individual conjugated bile acids, their quantitation by high performance liquid chromatography (HPLC) and 3α-hydroxysteroid dehydrogenase assay, and the possibility of their local synthesis. Improved methods of isolation are presented.

MATERIALS AND METHODS

All chemicals used were of analytical grade. Free and conjugated bile acids were obtained from Supelco, Bellefonte, PA, and the sulfated bile acids were purchased from P-L Biochemicals, Inc., Milwaukee, WI. Precoated thin-layer silica gel G plates of 0.5 mm thickness and silica gel 60 plates of 0.25 mm thickness were obtained from VWR Scientific, San Francisco, CA.

Rats were anesthetized with ether and the tissue sample e.g., heart, lung and kidney were excised and rinsed several times with physiological saline to remove traces of blood and then lyophilized. The equivalent of 2-4 gm of wet weight of tissue was used. The leucocytes were isolated from blood and the microsomes prepared by using the methods employed by Young and Rodwell [29].

Extraction Method I

One gram of lyophilized tissue was saponified with 15 ml of N NaOH in 90% ethanol at 65°C for 1 hr with occasional mixing. The sample was cooled, 5 ml of water was added and the neutral steroids were extracted twice with 15 ml of petroleum ether. The petroleum ether extract was backwashed with 5 ml of N NaOH in 50% ethanol to remove traces of bile acids and mixed with the original extract. Ten ml of 50% $ZnSO_4 \cdot 7H_2O$ were added to the petroleum ether extract and mixed thoroughly in a vortex mixer, then centrifuged for 10 min. at 3000 x g to remove most of the pigments. The supernate was decanted into a 500 ml round bottom flask and the precipitate was suspended in 15 ml of

95% ethanol containing 0.1% ammonium hydroxide and heated at 65°C for 30 min. The mixture was centrifuged again, the supernate decanted into the same round bottom flask and the precipitate was washed with 15 ml of 95% ethanol which also was added to the other washes. The pooled extract in the round bottom flask was evaporated to dryness in a rotary evaporator at 50-55°C under reduced pressure. The residue was then extracted with 20 ml of chloroform-methanol (1:1) which was evaporated almost to dryness under nitrogen in a water bath at 50-55°C. Any precipitate that came out was separated by centrifugation. The liquid that remained was subjected to TLC for the separation of fatty acids from bile acids.

Extraction Method II

One gram of lyophilized tissue was homogenized with 25-30 ml of 95% ethanol containing 0.5% HCl and incubated at 37°C for 1 hr [171]. The sample was centrifuged at 1000 x g for 10 minutes and the supernate was removed. Neutral steroids were extracted from the supernate with petroleum ether. The ethanolic residue was reduced in volume to 0.5 to 1 ml by evaporation in a rotary evaporator at 50°C.

Removal of Fatty Acids

Precoated silica gel G thin-layer chromatography (TLC) plates of 0.5 mm thickness were used. The extract was spotted as streaks on the plates and a standard solution containing a mixture of glycocholic, lithocholic and oleic acids spotted at one corner of the plate. The plate was developed in a filter paper-lined chamber containing the solvent system toluene:ethanol:methanol:water:ammonium hydroxide

(50:20:14:3:1) for 1 hr at room temperature (27-28°C). The plate was air dried under the hood and redeveloped in the same solvent system for one hour and the plate was dried in the oven. The entire plate except the known bile acid standard channel was covered with aluminum foil and the exposed area sprayed with a manganous chloride spray reagent [172]. The plate was heated in the oven at 100°C for 10 min. and the area between glycocholic and lithocholic acid was marked. An alternative color reagent was 10% phosphomolybdic acid in ethanol, with the plate heated at 105°C for less than 2 min. The bile acid area was scraped off the plate and eluted with chloroform-methanol (1:1). The extract was reduced to a volume of 0.5 ml under nitrogen at 50-55°C in a water bath.

TLC Separation of Bile Acids into Groups

Precoated plates of 0.25 mm thickness were used. The solvent system used was the same as reported earlier [173]. In practice, a standard solution containing a mixture of glycocholic, glycolithocholic, taurocholic, taurolithocholic, cholic and lithocholic acids was spotted at one corner of the plate and the unknown bile acid solution was spotted as streaks. The plate was run in the solvent system ethanol: isooctane:isopropyl alcohol:ethyl acetate (25:10:10:10) for 2.5 hr and then dried and the standard bile acid channel was sprayed with a color reagent. The glycine conjugated bile acids lay between glycocholic and glycolithocholic, taurine conjugated bile acids between taurocholic and taurolithocholic and free bile acids between cholic and lithocholic acid. The zone corresponding to the different bile acid groups was

scraped off the plates and the bile acids eluted with chloroform:methanol (1:1). The eluate was evaporated to a small volume for the identification of individual bile acids.

TLC Identification of Bile Acids

The individual bile acids were separated on TLC by different solvent systems e.g., (i) isooctane:ethyl acetate:acetic acid (10:10:2) [174], and hexane:methyl ethyl ketone: acetic acid (63:40:6) [175] for free bile acids and (ii) isoamyl alcohol:acetone: acetic acid:water (63:26:6:5) [176] and isoamyl acetate:propionic acid:n-propanol:water (4:3:2:1) [177] for glycine and taurine conjugated bile acids. The conjugated bile acids were hydrolyzed with 20% KOH in ethylene glycol and extracted through Amberlite XAD-2 column [178] and free bile acids were separated using TLC. The plate was sprayed with manganous chloride spray reagent and viewed under UV light and compared with authentic bile acids run on the same plate. With this reagent, less than a microgram of bile acids can be detected.

High Pressure Liquid Chromatography

Reverse phase high pressure liquid chromatography (HPLC) coupled with an ultraviolet detector set at 193 nm was used for identification and quantitation of conjugated bile acids [162]. Samples dissolved in the elution solvent mixture (2 propanol:8.8 mM potassium phosphate buffer pH 2.5, 160:340) were analyzed on a 4 mm x 30 cm μBondapak/C 18 column (Waters Associates) by eluting at 1 ml/min and 1 ml fractions were collected. Quantitation was done by comparing the sample peak area to corresponding standards.

Mass Spectrometry

HPLC and TLC fractionated sample components were collected in glass screw cap vials and concentrated to dryness. Twenty μl of solvent was added to each vial to redissolve the sample residue and 5 μl of each transferred to quartz capillary sample cups for solid probe mass spectrometry. The solvent was evaporated to dryness in the capillaries using a vacuum desiccator. Authentic compounds were used to obtain standard spectra.

Chemical ionization (CI) mass spectra were run using a Finnigan 4000 mass spectrometer and a Teknivent 29K interaction data system. Spectra were collected using the following conditions: 70 eV accelerating voltage, .025 torr source pressure CI methane, source temperature 260°C and manifold temperature 120°C.

Cholesterol 7α-hydroxylase

Leucocytes and rat tissues were analyzed for cholesterol 7α-hydroxylase activity. The sample was homogenized and microsomal preparations were made from the homogenates [29]. The microsomal pellet was resuspended in 1 ml of homogenizing buffer. Microsomal protein was determined by the Lowry procedure [160] and free cholesterol by the method of Allain et al. [179]. Cholesterol 7α-hydroxylase activity was measured using the modified Carlson et al. method [163]. Conversion of cholesterol to 7-hydroxy cholesterol in this system has been verified [180].

Enzymatic Assay of Total Bile Acids

Quantitation of bile acids in some samples was by a 3α-hydroxysteriod dehydrogenase (HSD) enzyme assay [181]. The 3α-hydroxysteroid dehydrogenase obtained from Sigma Chemicals was prepared in 0.01 M, pH 7.2 potassium phosphate buffer to give 3-4 units per ml. To 0.1 ml of sample or standard, 0.15 ml of 0.005 M βNAD and 0.6 ml of 0.1 M sodium pyrophosphate were added and the mixture was allowed to equilibrate at 25°C for 15 min. Absorbance at 340 nm was recorded; then 0.1 ml of enzyme solution was added and the solution was allowed to equilibrate for 40 min. The change in absorbance was recorded at 340 nm. The concentration of unknown was determined from the standard curve. The correlation of this analysis with GLC analysis has been verified [182].

RESULTS

Extraction

Method I was used for tissues subjected to TLC identification procedures. Method II was evaluated for quantitative recovery of added glycocholic acid (Table 1). From 88 to 93% of added radioactivity was recovered. The extraction method II [171] has been adopted as satisfactory for complete extraction.

TLC Isolation of Bile Acids

The TLC separation of bile acids from fatty acids is shown in Figure 1. It is evident from this figure that all the bile acids are clearly separated from fatty acids and cholesterol. Cholesterol moves far ahead of both bile acids and fatty acids. All bile acids, free or conjugated, lie between glycocholic and lithocholic acids except the sulfated bile acids. If sulfated bile acids are suspected to be in a biological specimen then glycolithocholic sulfate and lithocholic acid should be run as standards. All bile acids form a relatively narrow band which can be scraped from the plates after visualization with a color reagent. The solvent system should be freshly prepared and any remaining liquid should be drained from the chamber before fresh solvent is added. This solvent system is very useful to isolate the bile acids in their biological state without destroying the structure. The recovery of bile acids from the thin layer chromatogram by enzymatic assay was more than 90%.

Table 1. Recovery of added glycocholic acid by extraction method II

Tissue	^{14}C-glycocholic acid		
	added	recovered	
	cpm	cpm	%
liver	13242	11918	90
heart	11849	10901	92
lung	11769	10945	93
kidney	12879	11720	91
adipose	13018	11456	88

Figure 1. Thin-layer chromatography on silica gel G of 0.5 mm thickness developed with toluene/ethanol/methanol/water/ammonium hydroxide (50:20:14:3:1,v/v/v/v/v) and visualized with manganous chloride spray reagent. 1 = glycocholic lithocholic and oleic acid (bottom to top); 2 = glycolithocholic sulfate; 3 = taurolithocholic acid; 4 = taurolithocholic sulfate; 5 = lithocholic sulfate; 6 = glycolithocholic acid; 7 = glycochenodeoxycholic acid; 8 = taurocholic acid; 9 = taurochenodeoxycholic acid; 10 = cholic acid; 11 = hyocholic acid; 12 = hyodeoxycholic acid; 13 = chenodeoxycholic acid; 14 = cholesterol; 15 = glycocholic glycolithocholic, taurocholic, taurolithocholic, cholic, lithocholic and oleic acid.

TLC Identification of Bile Acids in Tissues

The results of TLC identification of bile acids in heart, lung and kidney are illustrated by Figure 2. It appears that cholic acid is present in each tissue examined by TLC in the solvent system isooctane: ethyl acetate:acetic acid (10:10:2). It is apparent from Figure 2 that chenodeoxycholic acid is predominant in lung while cholic acid is most abundant in heart and kidney. Traces of material were detected in bands corresponding to hyodeoxycholic and lithocholic acids.

Physical Identification of Bile Acids

The glycine conjugated bile acid spectra made using low ionizing voltages were quite similar to published spectra [183] (Table 2). The molecular ion and some other higher mass ions were not evident in the spectra. This was attributed to the small sample size and also possible interference from dissolved silica from the TLC plates.

Attempts to improve the spectra by using chemical ionisation mass spectrometry were successful to a limited extent. The spectra generated did not resemble published EI spectra. However, the spectra of the reference standards and the isolated samples were virtually identical, thus, helping to establish the identity of the bile acids (Table 3).

Neither the EI nor the CI spectra of the taurine conjugated bile acids closely matched the spectra published in the literature (Table 4). We thought that samples were thermally degraded in the sample probe. Spectra of the commercial standards and the isolated samples were almost identical to each other, however, and contribute to identifying the samples.

Figure 2. Thin-layer chromatography on silica gel 60 of 0.25-mm thickness developed with isooctane/ethyl acetate/acetic acid (10:10:2,v/v/v) and visualized with manganous chloride spray reagent and UV light. 1 = cholic, hyodeoxy cholic, chenodeoxycholic and lithocholic acid; 2 = heart; 3 = same as 1; 4 = kidney; 5 = same as 1; 6 = lung; 7 = same as 1. Top large bands in 2, 4 and 6 are pigment impurities.

Table 2. Comparison of authentic glycine conjugated cholanoic acid electron impact spectra with published data and with a tissue compound isolated by TLC

	Glycocholic Acid		
m/e	Reference 183	Standard	Rat Kidney
465	.4	----	----
429	8.0	----	----
414	4.3	----	----
411	8.8	----	----
396	6.2	----	----
390	2.3	----	----
372	12.3	6.8	.8
354	13.7	13.0	1.6
336	2.9	----	----
312	6.8	6.2	1.2
299	5.0	----	.5
294	6.3	----	1.4
281	7.5	----	1.8
271	90.6	73.8	71.6
253	100.0	57.8	70.6
244	8.8	1.6	4.9
226	21.0	9.2	14.2
211	15.5	4.0	10.4
199	18.1	7.9	11.3
147	23.6	5.0	17.4
145	28.1	7.8	28.2
130	15.2	38.2	26.9
117	32.1	100.0	42.1
76	17.5	13.9	7.6
30	36.6	NA	NA
449	1.5	----	----
431	8.6	24.4	----
416	6.5	----	----

Table 2. (Continued)

	Glycocholic Acid		
m/e	Reference 183	Standard	Rat Kidney
413	30.9	42.8	----
398	45.2	75.8	----
374	6.8	9.3	----
356	7.9	64.3	----
338	3.7	3.1	----
314	15.1	52.1	----
401	2.5	3.1	----
296	7.7	2.1	----
283	3.8	.8	----
273	18.6	7.7	----
255	44.7	20.0	----
246	8.2	3.4	----
228	14.2	6.7	----
213	31.6	12.6	----
201	18.1	7.6	----
147	24.8	3.5	----
145	21.6	12.1	----
130	46.3	37.1	----
117	100.0	100.0	----

Table 3. Comparison of authentic glycine conjugated cholanoic acid chemical ionization spectra with tissue samples isolated by TLC

Glycochenodeoxycholic Acid			Glycocholic Acid		
m/e	Standard	Adipose Tissue	m/e	Standard	Adipose Tissue
413	2.8	1.8	412	4.8	3.9
398	0.7	-	411	8.9	-
			373	6.3	3.8
356	11.3	5.3	372	5.6	-
338	4.1	3.5	354	24.2	14.0
			353	26.4	-
296	0.8	0.8	337	45.3	30.2
283	4.8	3.3	319	7.8	-
273	1.1	0.5	271	-	3.8
255	45.4	52.9	253	13.2	14.0
228	6.7	6.9	211	5.2	6.0
213	13.5	13.5	199	4.6	5.0
201	11.5	8.2	147	4.9	6.4
147	17.2	15.1	145	5.4	7.8
145	18.4	17.7	130	7.4	27.4
130	5.6	6.1	117	29.8	36.4
117	14.8	14.2			

Table 4. Comparison of authentic taurine conjugated cholanoic acid chemical ionization spectra with tissue samples isolated by HPLC

m/e	Taurocholic Acid			m/e	Taurochenodeoxycholic Acid		
	Reference 183	Standard	Human Leucocyte		Reference 183	Standard	Human Leucocyte
379	2.8	-	-	385	-	4.6	-
353	16.7	13.1	4.6	381	2.5	-	-
338	6.3	25.6	9.2	357	-	100.0	47.4
337	-	100.0	29.6	355	100.0	25.6	13.8
335	6.9	98.0	51.3	340	55.9	5.3	11.8
320	4.9	30.9	3.9	339	-	21.7	53.2
312	0.6	-	-	337	14.9	-	-
299	5.9	-	-	322	10.0	-	-
295	2.8	-	-	314	2.7	-	-
294	5.0	-	-	301	42.0	50.0	-
271	0.9	-	-	297	4.3	-	-
253	100.0	12.5	9.9	296	5.1	-	-
244	0.9	-	-	283	12.4	-	-
243	-	2.6	2.6	273	4.0	-	-
226	6.1	2.6	4.6	257	-	9.9	19.7
211	6.4	3.9	3.9	255	80.6	9.2	13.1
125	-	61.8	63.1	246	3.5	-	-
				229	-	11.8	11.8
				228	11.5	-	-
				213	48.3	5.3	6.5
				127	-	71.0	75.7
				113	-	98.0	100.0

Quantitation of Bile Acids

HPLC quantitation was compared to HSD assay. Results indicate similarity between HSD and HPLC quantitations (Table 5). Rat heart, lung, kidney and adipose tissue as well as liver contained cholanoic acids (Table 6). They were predominantly taurine conjugated in the animals used (adult Sprague-Dawley male rats fed laboratory ration). Glycine conjugated compounds were present and could be concentrated by TLC as shown by the mass spectra data. Human leucocytes contained bile acids (Table 6). Glycine conjugated cholic acid (GC) was predominant, while for chenodeoxycholic acid, the taurine conjugated form was most abundant.

Cholesterol 7α-hydroxylase Analyses

The presence of cholesterol 7α-hydroxylase was demonstrated in non-hepatic rat tissue as shown in Table 7. Heart, lung and kidney tissues exhibited significant levels of activity; 23 to 32% of the level found in liver. Human mixed leucocytes had about 10% of the activity of rat liver per mg of microsomal protein.

Table 5. Correlation of HPLC and 3α-hydroxysteroid dehydrogenase (HSD) of cholanoic acids in rat tissues. r = 0.945

	HPLC	HSD
Tissue	Total Cholanoic Acids	
	μg per g tissue	
liver	165	153.4
heart	13.9	10.8
lung	11.8	10.9
kidney	32.3	31.2
adipose	5.2	4.4

Table 6. HPLC quantitation of cholanoic acids in nonhepatic tissues

Tissue	Total	TC[a]	TCDC[a]	TDC[a]	GC[a]	GCDC[a]	GDC[a]
Rat (μg/g tissue)							
liver	165	115	49.5	ND[b]	ND	ND	ND
heart	13.9	9.7	4.2	ND	ND	ND	ND
lung	11.8	4.4	7.4	ND	ND	ND	ND
kidney	32.3	15.4	11.3	3.4	2.2	ND	ND
adipose	8.2	2.4	3.4	2.4	ND	ND	ND
Human (μg/ml blood)							
mixed leucocytes	1.5	0.49	0.13	ND	0.57	0.31	0.04

[a]TC = Taurocholic; TCDC = Taurochenodeoxycholic; TDC = Taurodeoxycholic; GC = Glycocholic; GCDC = Glycochenodeoxycholic; GDC = Glycodeoxycholic acids.

[b]Not detectable.

Table 7. Cholesterol 7α-hydroxylase activity microsomes of selected tissues

Tissue	% Cholesterol converted to 7α-hydroxycholesterol	NMOLS of 7α-hydroxy-cholesterol formed
	per mg microsomal protein	per 40 mm
liver	4.56	1.026
heart	1.45	0.203
lung	1.03	0.241
kidney	1.34	0.378
mixed leucocytes	0.247	0.113

DISCUSSION

The identification of conjugated bile acids obtained by HPLC and TLC isolation from tissues validates the use of HPLC and other methods in which comparison to standards is used for identification of bile acids. The methods presented herein constitute relatively simple and effective procedures for isolation and quantitation of known bile acids from biological sources. For total bile acids, the HSD method is rapid and for individual bile acids HPLC allows identification and quantitation without the use of harsh deconjugation procedures.

The presence of cholanoic acids in all tissues examined requires explanation. These compounds are toxic under some conditions [184] and are not likely to accumulate naturally without protective processes. Our data on the presence of cholesterol 7α-hydroxylase activity suggest that the compounds may originate in the tissue analyzed and not from blood circulation. Further verification of this with identification of intermediates is required.

Bile acids have been known to be required for pancreatic cholesterol esterase activity for many years [185]. A recent publication [186] describes a requirement for bile salts for human carboxyl ester hydrolase from pancreatic juice to hydrolyze esters of cholesterol and vitamins A, D-3, and E.

In a review of cholesterol efflux from cells, Phillips et al. [187] concluded that a large unstirred water layer affects the transfer of cholesterol between cells and acceptors in a medium. Bile acids are obviously suitable mediators of lipid transport across unstirred water

layers [188].

In addition to possible functions of cholanoic acids as mediators of sterol efflux from cells through the unstirred water layer, cholesterol efflux requires that the cholesterol be in the free form [189]. Any stored cholesterol ester must first be hydrolyzed to cross the membrane. Regulation of hydrolysis and esterification could be mediated by cholanoic acids. Hydroxylated sterols are known to be potent activators of cholesterol esterase [190]. Cholesterol synthesis has been shown to be required for leucocyte chemotaxis [191]. Further investigation of the possible functions of cholanoic acids in non-hepatic tissues is warranted.

PAPER III. EFFECT OF AGE AND DIET COMPOSITION ON BLOOD PRESSURE AND CHOLESTEROL METABOLISM IN PLASMA LEUCOCYTES AND LIPOPROTEINS IN MEN

Effect of age and diet composition on blood pressure and cholesterol metabolism in plasma leucocytes and lipoproteins in men

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ABSTRACT

The effects of age and diet composition on the cholesterol metabolism in plasma leucocytes and lipoproteins of healthy men of three different age groups (19-25, 40-50 and 60-70 yrs) were studied. The cholesterol levels in the leucocytes, plasma, VLDL and LDL of the 60-70 year old men were significantly higher than those of the two younger groups, whereas HDL levels were very similar in all the three age groups. The mean HMGR and COH activity in the two older groups was significantly higher than in that of 19-25 year old men. With increase in age, plasma and VLDL triglycerides increased significantly, whereas no significant changes in phospholipid and bile acid levels in plasma or lipoproteins were observed. The dietary intake of calories, carbohydrates and fat in 19-25 year old men differed significantly from 40-50 year old men, but not from the 60-70 year old men. Most of the dietary variables examined had no significant effect on plasma lipids. The results suggest that diet is not responsible for the age related increase in plasma leucocyte and lipoprotein lipids. The increase in plasma cholesterol in the oldest age group may reflect a decrease in number or function of the peripheral cell receptors.

INTRODUCTION

The dominant role that coronary heart disease (CHD) plays in the cause of death in this country underscores the importance of atherosclerosis as a population health problem. The incidence increases with age, from 2.3% at 15-24 years of age to 44.0% at 65 years of older [1]. That high serum cholesterol levels are implicated in atherosclerotic progression is indisputable. The lipoproteins which transport cholesterol have also been indicted in the development of atherosclerosis. The low density lipoproteins (LDL) carry cholesterol to the peripheral tissues where it is used and the high density lipoproteins (HDL) may return cholesterol from peripheral tissues back to the liver. Epidemiological evidence suggests that high concentrations of LDL increase the risk of CHD, whereas high concentrations of HDL decrease that risk [192]. Many studies have reported a rise in serum and LDL cholesterol and a decrease in HDL cholesterol as age increases [58,61,65]. Juustila [45] observed that systolic hypertension was accompanied by high serum cholesterol concentrations. Six decades of evidence have provided a considerable degree of certainty that the level of plasma and lipoprotein cholesterol is determined partially by the intake of calories, fat and cholesterol [193]. However, it is still not known to what extent diet is responsible for the age-related changes in plasma and lipoprotein cholestrol.

The atherosclerotic plaques are characterized by the accumulation of cholesterol and its esters in the peripheral tissues. These deposits could come from three sources namely, increased influx from blood mediated by an increase in LDL; excess cholesterol synthesis in peripheral

tissues; and decreased efflux from peripheral cells mediated by HDL. This stresses the importance of understanding the regulation of cholesterol synthesis and utilization by peripheral tissues in vivo. The usefulness of leucocyte for studying cholesterol biosynthesis in peripheral tissues has been reported by Fredrickson and Sloan [12], Hsia [14] and Fogelman et al. [13]. We have evidence that the leucocytes can also synthesize bile acids, the end product of cholesterol catabolism [180]. An indicator of cholesterol metabolism in peripheral tissues is therefore envisioned in the leucocyte. It is believed that the leucocyte will reflect in vivo responses of peripheral tissue to factors affecting cholesterol metabolism. Therefore, the present study was designed to use leucocytes for investigating the effects of age and diet composition on cholesterol metabolism in peripheral tissues of adult men.

MATERIALS AND METHODS

Selection and Description of Subjects

The subjects for this study were 29 men from three different age groups (19-25, 40-50, and 60-70 years) who were students, and current and former employees at Iowa State University. All were white (Caucasian background) and in apparent good health. None of the subjects were engaged in regular jogging, swimming or other training exercises. Information describing these men is given in Table 1.

Dietary Data, Blood Pressure and Blood Sample Collection

The subjects were instructed how to record their food intake in household measures for three consecutive days which consisted of two weekdays and one day of the weekend. The diet records were analyzed for their energy and nutrient content by using a computer program based on food values in the Agriculture Handbook, 456 [194]. Within the same week diet records were kept, a 100 ml sample of fasting blood was drawn once by venipuncture in EDTA vacutainer tubes. Blood pressure was recorded before drawing blood by using sphygnomanometer.

Laboratory and Data Analyses

A 30 ml blood sample was used for the separation of mixed leucocytes [29], 30 ml for separation of mononuclear leucocytes [159], and the rest of the sample for plasma and lipoprotein separations [195]. The number of cells was determined by coulter counter and differential count with Wright stain [196]. Mixed leucocytes were lyophilized and extracted with chloroform: methanol (1:1 v/v). The biochemical tests performed on plasma,

lipoproteins and mixed leucocytes included determination of cholesterol (free and ester), total bile acids, phospholipids and triglycerides. Total and free cholesterol concentrations were determined by the method of Allain et al. [179] and cholesterol ester represented the difference between total and free cholesterol. Total bile acids were analyzed by the method of Turnberg and Anthony-Mote [181]; phospholipids by using the method of Sandhu [197], and triglycerides by the method of Fletcher [198]. Microsomes from mononuclear cells were prepared by the method of Young and Rodwell [29] and HMGCoA reductase activity was measured in microsomes also using Young and Rodwell's procedures. Cholesterol 7α-hydroxylase activity was measured in mononuclear microsomes using the modified Carlson et al. method (163). The methods used are included in Appendix B.

Paired Student t-tests were performed on the dietary, clinical and biochemical data to examine effects of age. Correlation coefficients between selected variables were also calculated.

RESULTS

General Characteristics

Table 1 shows the characteristics of the subjects studied. They ranged from 19-68 years of age and were divided into three age groups: 19-25 years, 40-50 years, 60-70 years. The mean weights and relative weights of the subjects in the three age groups did not differ significantly. The mean systolic pressure of 60-70 year old men was significantly greater than those for the 19-25 and 40-50 year old men, but the diastolic pressure of men in the three age groups did not differ significantly.

Dietary Composition

Diet composition data (Table 2) show that the total calories, fat, protein, and carbohydrate intakes of 19-25 year old men differed significantly from the 40-50 year old men, but not from the 60-70 year old men. The mean saturated and the polyunsaturated fatty acid intakes of the two older groups studied were significantly lower than that of the 19-25 year old group of men, while the polyunsaturated to saturated ratios of the diets were similar among the three groups. The percentages of calories derived from fat, protein and carbohydrate were not significantly different among the three age groups.

Leucocytes

There was no significant difference in the number or proportion of leucocytes as age increased (Table 3). However, the cholesterol content of leucocytes of the oldest age group studied was significantly higher

than the mean values for the two younger groups. The mean total bile acid levels were similar in all three age groups.

Our previous work [180] has shown that the mononuclear leucocytes possess hydroxymethylglutaryl coenzyme A reductase (HMGR) and cholesterol 7α-hydroxylase activity (COH). Hydroxymethylglutaryl coenzyme A reductase is the rate-limiting step in the synthesis of cholesterol [199]. The mean HMGR activity for the 40-50 and 60-70 year old men was significantly higher than that for the youngest age group when activity was expressed as per mg microsomal protein or per 10^7 mononuclear cells or per ml blood (Table 4). Cholesterol 7α-hydroxylase is the rate limiting step of bile acid synthesis [93]. When expressed as per mg microsomal protein or per ml blood, its activity was similar for the three age groups. But when values were expressed as per 10^7 mononuclear cells, the COH activity for the 40-50 and 60-70 year old men was significantly higher than that observed in the 19-25 year old men. This particular trend reflects the decrease in the proportion of mononuclear cells with increase in age.

Plasma

Table 5 shows the concentration of various lipids in plasma. The mean plasma cholesterol values increased significantly with increases in age from 19 to 70 years and the mean annual change was 1.57 mg cholesterol per dl plasma. The magnitude of increase in plasma free cholesterol and cholesterol ester with age was similar, consequently free to ester ratio did not change with increased age. For the plasma phospholipid, mean values were similar in all three age groups, although a

significant increase in cholesterol to phospholipid ratio with increase in age was observed. Plasma triglycerides increased significantly for the successive age groups with 93 mg/dl in the 19-25 group, 107 mg/dl in the 40-50 group and 122 mg/dl in the 60-70 group. The mean values for plasma bile acids did not differ significantly among the three age groups.

Lipoproteins

The levels of various lipids in the different lipoproteins are shown in Table 6. The change in VLDL total cholesterol with advancing age was not significant, while its free cholesterol was significantly higher in the 60-70 age group than in the 19-25 age group. The LDL cholesterol increased significantly with increase in age at a mean rate of 1.29 mg per dl plasma per year. The degree of rise in LDL cholesterol ester was proportionately greater than the increments in its free cholesterol from 19-25 to 40-50 years, but the increase in both free and ester was significant from 40-50 to 60-70 years. There were no significant changes in HDL cholesterol and in the free to ester ratio.

All three age groups showed similar mean phospholipid concantrations in all the lipoprotein fractions analyzed. VLDL triglycerides increased significantly with advancing age, while LDL and HDL triglycerides remained unchanged. Bile acids were found to be present in all the three lipoprotein fractions, LDL, VLDL and HDL, and these amounts accounted for almost all the plasma bile acids. The lipoprotein bile acids tended to decrease with increase in age, but this trend was not significant.

Correlation coefficients between selected variables are shown in Tables 7 and 8. These are discussed later in the paper.

Table 1. Characteristics of men of 3 age groups (mean and range)

Variable	Age Groups, yr 19-25 n=10	40-50 n=9	60-70 n=10
Age (yrs)	22.4 (19-25)	43.8 (40-50)	64.0 (61-68)
Weight (lbs)	175.8[a][1] (143-199)	173.8[a] (136-208)	172.6[a] (127-195)
Relative weight[2]	99.5[a] (83-109)	98.3[a] (83-108)	102.3[a] (78-120)
Blood Pressure (mm Hg)			
Systolic	118.4[a] (94-152)	115.5[a] (88-140)	136.6[b] (120-156)
Diastolic	70.2[a] (56-84)	76.7[a] (62-98)	78.8[a] (58-96)

[1]Means with different letter superscripts within the same row are significantly different ($p < 0.05$ or $p < 0.01$).

[2]Relative weight, % = $\frac{\text{Actual weight}}{\text{Desirable weight for height}} \times 100$

Table 2. Diet composition of healthy men in 3 different age groups (Mean of 3 days ± Standard error of mean)

Variable	Age Groups, yr 19-25 n=10	40-50 n=9	60-70 n=10
1) Protein(g)	102[a][1] ± 9	77[b] ± 10	106[a] ± 17
2) Carbohydrate (g)	310[a] ± 27	208[b] ± 24	258[ab] ± 21
3) Fat(g)			
Total	120[a] ± 9	83[b] ± 10	94[ab] ± 13
Saturated	49[a]	30[b]	34[b]
Oleic	45[a]	30[b]	34[b]
Linoleic	21[a]	13[b]	14[b]
P/S[2]	0.402[a]	0.451[a]	0.443[a]
4) Cholesterol(mg)	456[a] ± 68	335[b] ± 61	415[c] ± 68
5) Calories			
Total	2763[a] ± 179	1927[b] ± 223	2322[ab] ± 197
Protein (%)	15[a]	16[a]	18[a]
Carbohydrate (%)	46[a]	45[a]	46[a]
Fat (%)	39[a]	39[a]	36[a]

[1]Means with different letter superscripts within the same row are significantly different ($p < 0.05$ or $p < 0.01$).

[2]Polyunsaturated : saturated fatty acid ratio.

Table 3. Total and differential leucocyte count, and cholesterol and bile acid concentrations in leucocytes of healthy men in 3 different age groups (Mean ± Standard error of mean)

Variable	Age Groups, yr 19-25 n=10	40-50 n=9	60-70 n=10
Leucocytes			
Total number of leucocytes	7194[a][1] ± 474	6494[a] ± 417	7816[a] ± 694
Mononuclear leucocytes (%)	44[a] ± 2.7	40[a] ± 3.4	38[a] ± 2.9
Granulocytes (%)	56[a] ± 2.7	60[a] ± 3.4	62[a] ± 2.9
Cholesterol (µg per 10^8 cells)			
Total	101[a] ± 11.9	106[a] ± 14.3	135[b] ± 28.1
Free cholesterol	78[a] ± 10.3	79[a] ± 10.8	100[b] ± 18.9
Cholesterol ester	23[a] ± 3.1	27[a] ± 3.8	35[a] ± 9.4
Bile acids (nMoles per 10^8 cells)	2.11[a] ± 0.164	2.16[a] ± 0.252	2.11[a] ± 0.178

[1]Means with different letter superscripts within the same row are significantly different ($p < 0.05$ or $p < 0.01$).

Table 4. Hydroxymethylglutaryl coenzyme A reductase (HMGR)[1] and cholesterol 7α-hydroxylase (COH)[2] activity in mononuclear leucocyte microsomes of healthy men in 3 different age groups (Mean ± Standard error of mean)

	Age Groups, yr					
	19-25 n=10		40-50 n=9		60-70 n=10	
	HMGR	COH	HMGR	COH	HMGR	COH
Per mg microsomal protein	10.5[a][3] ± 0.54	0.106[a] ± 0.0059	13.1[b] ± 0.81	0.107[a] ± 0.0051	14.4[b] ± 1.22	0.128[a] ± 0.0113
Per 10^7 mononuclear cells	30.4[a] ± 3.24	0.303[a] ± 0.0300	51.4[b] ± 6.52	0.419[ab] ± 0.0549	45.2[b] ± 4.10	0.401[b] ± 0.0386
Per ml blood	9.1[a] ± 0.49	0.091[a] ± 0.0054	12.2[b] ± 0.75	0.099[a] ± 0.0079	12.5[b] ± 0.77	0.110[a] ± 0.0069

[1]Enzyme activity was expressed as p mols of mevalonate formed per hour.

[2]Enzyme activity was expressed as % of ^{14}C-cholesterol converted to ^{14}C-7α-hydroxycholesterol per 40 minutes.

[3]Means with different letter superscripts within the same row are significantly different ($p < 0.05$ or $p < 0.01$).

Table 5. Cholesterol, phospholipid, triglyceride and bile acid levels in plasma of healthy men in 3 different age groups (Mean ± Standard error of mean)

Variable[1] (per dl plasma)	Age Groups, yr 19-25 n=10	40-50 n=9	60-70 n=10
TC (mg)	204[a][2] ± 5.4	232[b] ± 11.1	283[c] ± 8.3
FC (mg)	53[a] ± 1.8	62[b] ± 4.2	74[c] ± 2.1
CE[3] (mg)	150[a] ± 3.9	170[b] ± 7.2	209[c] ± 7.1
PL (mg)	210[a] ± 20.5	208[a] ± 10.7	215[a] ± 21.7
TG (mg)	93[a] ± 3.5	107[b] ± 7.2	122[c] ± 5.5
BA (μM)	0.749[a] ± 0.077	0.767[a] ± 0.094	0.609[a] ± 0.056
FC/CE	0.35[a]	0.36[a]	0.35[a]
TC/PL	0.97[a]	1.11[b]	1.32[c]

[1]TC = Total cholesterol, CE = Cholesterol ester, FC = Free cholesterol PL = Phospholipids, TG = Triglycerides, and BA = Bile acids.

[2]Means with different letter superscripts within the same row are significantly different ($p < 0.05$ or $p < 0.01$).

[3]Calculated CE = Total cholesterol minus free cholesterol.

Table 6. Cholesterol, phospholipid, triglyceride and bile acid levels in lipoproteins of healthy men in 3 different age groups[1] (Mean ± Standard error of mean)

	Age groups (yr)	TC (mg)	FC (mg)	CE[2] (mg)	PL (mg)	TG (mg)	BA (μM)	FC/CE	TC/PL
		per dl plasma							
Very low density lipoprotein	19-25	20.4[a][3] ± 0.97	4.7[a] ± 0.24	15.7[a] ± 0.89	33.2[a] ± 2.20	54.5[a] ± 2.23	0.105[a] ± 0.007	0.30[a]	0.61[a]
	40-50	21.3[a] ± 2.35	5.3[ab] ± 0.67	17.0[a] ± 1.46	29.8[a] ± 1.47	64.4[b] ± 3.08	0.104[a] ± 0.006	0.31[a]	0.71[ab]
	60-70	24.9[a] ± 2.09	6.5[b] ± 0.57	18.4[a] ± 1.53	30.3[a] ± 2.36	83.7[c] ± 4.18	0.097[a] ± 0.055	0.35[a]	0.82[ab]

[1]TC = Total cholesterol, CE = Cholesterol ester, FC = Free cholesterol, PL = Phospholipids, TG = Triglyceride, BA = Bile acids.

[2]Calculated CE = Total cholesterol minus free cholesterol.

[3]Mean values not sharing a common letter superscript are significantly different ($p < .05$ or $p < .01$).

Table 6. (Continued)

	Age groups (yr)	TC (mg)	FC (mg)	CE[2] (mg)	PL (mg)	TG (mg)	BA (μM)	FC/CE	TC/PL
		per dl plasma							
Low density lipoprotein	19-25	127.1[a] ± 5.45	30.4[a] ± 1.43	96.7[a] ± 4.44	121.9[a] ± 17.39	24.4[a] ± 0.90	0.423[a] ± 0.051	0.31[a]	1.04[a]
	40-50	145.4[b] ± 6.16	30.7[a] ± 2.22	114.7[b] ± 5.09	118.4[a] ± 8.62	26.7[a] ± 1.41	0.402[a] ± 0.043	0.27[a]	1.38[c]
	60-70	193.1[c] ± 6.04	41.4[b] ± 1.48	151.7[c] ± 5.77	139.6[a] ± 18.29	24.6[a] ± 1.30	0.317[a] ± 0.037	0.27[a]	1.38[c]
High density lipoprotein	19-25	47.8[a] ± 1.89	10.9[a] ± 0.34	36.9[a] ± 1.71	54.0[a] ± 3.06	13.1[a] ± 0.90	0.216[a] ± 0.016	0.29[a]	0.88[a]
	40-50	46.6[a] ± 1.27	11.7[a] ± 0.77	34.9[a] ± 1.24	52.3[a] ± 2.03	13.1[a] ± 0.94	0.198[a] ± 0.013	0.31[a]	0.89[a]
	60-70	46.3[a] ± 2.55	9.8[a] ± 0.55	36.5[a] ± 2.16	50.0[a] ± 2.06	13.2[a] ± 0.89	0.189[a] ± 0.012	0.27[a]	0.95[a]

Table 7. Correlations coefficients between selected variables

Variables	Age Groups (yr) 19-25 n=10	40-50 n=9	60-70 n=10	All 19-70 n=29
	Correlation Coefficients			
Age				
Systolic	-0.23	-0.01	0.32	0.45*
Diastolic	-0.36	0.30	0.33	0.35
Plasma Cholesterol	0.42	-0.26	-0.15	0.78**
LDL cholesterol	0.06	0.16	-0.16	0.84**
HMGCoA reductase	0.11	-0.67	0.26	0.49**
Cholesterol 7α-hydroxylase	0.08	-0.43	0.58	0.37*
Plasma Triglyceride	0.09	0.05	0.04	0.68**
VLDL Triglyceride	0.27	-0.21	-0.13	0.76**
Systolic & Diastolic	0.67*	0.81**	0.56	0.64**
Plasma Cholesterol	0.23	0.46	0.02	0.52**
Systolic	0.23	0.46	0.02	0.52**
Mononuclears (%)	0.01	-0.43	-0.49	-0.42*
HMGCoA reductase	0.80**	0.66*	0.54	0.69**
Cholesterol 7α-hydroxylase	0.76**	0.63	0.37	0.55**
VLDL cholesterol	0.14	0.57	0.13	0.39*
LDL cholesterol	0.32	0.14	0.91**	0.83**
HDL cholesterol	0.25	-0.19	-0.22	-0.08
Plasma Triglyceride	-0.16	0.36	0.79**	0.73**
VLDL Triglyceride	0.08	0.47	0.79**	0.83**
Systolic				
HMGCoA reductase	0.37	0.39	0.55	0.48**
Cholesterol 7α-hydroxylase	0.42	C.64	0.65*	0.62**

*P < 0.05.

**P < 0.01

Table 7. (Continued)

Variables	Age Groups (yr) 19-25 n=10	40-50 n=9	60-70 n=10	All 19-70 n=29
	Correlation Coefficients			
LDL cholesterol				
Systolic	0.33	0.48	-0.26	0.54**
HMGCoA reductase	0.21	0.12	0.49	0.54**
Cholesterol 7α-hydroxylase	0.53	0.01	0.28	0.46**
Plasma triglyceride	-0.19	-0.31	0.62*	0.63**
VLDL triglyceride	-0.13	-0.01	0.66*	0.77**
Plasma triglyceride				
VIDL triglyceride	0.96**	0.88**	0.95**	0.95**
LDL triglyceride	0.59	0.41	0.69**	0.39*
HDL triglyceride	0.68*	0.60	0.74**	0.50**

Table 8. Correlation coefficients between selected dietary variables, blood pressure, plasma leucocyte and lipoprotein lipids.

Variable	Age Groups (yr) 19-25 n=10	40-50 n=9	60-70 n=10	All 19-70 n=29
	Correlation Coefficients			
Calories				
Systolic	-0.37	-0.45	-0.60	-0.30
Diastolic	-0.23	-0.44	-0.35	-0.41*
Plasma TC	-0.39	-0.43	0.08	-0.27
VLDL TC	-0.79**	-0.26	-0.24	-0.37*
VLDL TG	-0.61	-0.17	0.09	-0.21
Carbohydrate				
Systolic	-0.47	-0.55	-0.28	-0.35
Diastolic	-0.10	-0.68*	-0.25	-0.39*
Plasma TC	-0.43	-0.43	0.06	-0.28
Plasma TG	-0.59	-0.06	-0.49	-0.41*
VLDL TC	-0.79**	-0.31	-0.53	-0.46**
VLDL TG	-0.68*	-0.04	0.43	-0.33
LDL BA	0.66*	0.25	0.39	0.47**
Fat				
Weight	0.37	0.46	0.39	0.38*
Leucocyte TC	-0.12	-0.25	-0.55	-0.40*
Plasma TC	0.02	-0.38	0.07	-0.24
Plasma TG	-0.53	-0.44	0.11	-0.29
VLDL TC	-0.79**	0.02	-0.01	-0.19
VLDL TG	-0.43	-0.29	0.34	-0.15
VLDL BA	0.42	0.34	0.58	0.43*
LDL BA	0.65*	0.31	0.07	0.36*
HDL BA	0.44	0.29	0.53	0.46*

*$P < 0.05$.

**$P < 0.01$.

Table 8. (Continued)

Variable	Age Groups (yr) 19-25 n=10	40-50 n=9	60-70 n=10	All 19-70 n=29
	Correlation Coefficients			
Protein				
Plasma TC	-0.05	- 0.43	-0.32	-0.09
Plasma TG	-0.41	- 0.35	-0.12	-0.12
Cholesterol				
Mononuclear (%)	0.62	0.37	0.74**	0.57**
Plasma TC	0.004	0.21	-0.49	-0.09
VLDL TC	-0.17	0.05	0.16	0.02
LDL TC	0.70*	-0.03	-0.55	-0.06
HDL TC	-0.18	-0.05	0.52	0.19
Saturated Fat				
Mononuclear (%)	0.57	0.14	0.81**	0.58**
Plasma TC	0.08	-0.03	-0.56	-0.39*
Plasma TG	-0.26	0.04	-0.44	-0.42*
VLDL TG	-0.25	0.07	-0.37	-0.37*
Polyunsaturated Fat				
Mononuclear (%)	0.26	0.32	0.58	0.39*
Plasma TC	0.08	-0.07	0.002	-0.19
Plasma TG	-0.35	0.33	-0.31	-0.35
VLDL TG	-0.51	0.39	-0.17	-0.29
LDL TG	-0.49	-0.06	-0.59	-0.39*

DISCUSSION

We observed a significant increase in systolic blood pressure with increase in age and our results are consistent with the findings of the HANES study [46]. The diastolic pressure also increased with age but not to a significant degree. As expected, analysis of our data (Table 7) has shown a high degree of correlation between systolic and diastolic pressure levels (0.64). An interdependence between serum cholesterol and blood pressure has often been suggested, but the relationship has never been clearly shown in man. Our results show significant and positive relationships of plasma and LDL cholesterol (free and ester) with systolic blood pressure and these trends are in agreement with the findings of Juustila [45]. Adel et al. [48] observed that *in vivo*, hypertensive rats synthesize cholesterol more rapidly than do normotensive rats. Our results also show that the HMGCoA reductase activity was high in men with high systolic or diastolic pressure and this positive association was significant.

No change in the total leucocyte count occurred with aging and this is consistent with the observations of Helman and Rubenstein [30], Otani [32] and Zacharski et al. [33]. We observed a decline in the proportion of mononuclear cells with increase in age and this trend has also been reported by Mackinney [34] and Reddy and Goh [35]. Friedman et al. [19] have reported the use of leucocyte count as a predictor of myocardial infarction and their data suggest that the predictive power of leucocyte count is quite similar to that of serum cholesterol or blood pressure. We did not observe any association between the total leucocyte

count and the plasma cholesterol or other lipids or blood pressure.

In our study, the HMGCoA reductase activity in the mononuclear cells increased significantly as men aged from 19 to 50 years which was followed by little change in the 60-70 years. Our results do not agree with the observations in rats by Yamamoto and Yamamura [90], Story and Kritchevsky [95] and Dupont et al. [96] who reported either a decrease or no change in cholesterol synthesis in liver and other extrahepatic tissues as the animals aged. The discrepancy may be attributed partly to differences in the tissue studied or in the response of humans and rats. The significant and positive association of both HMGR ($r = 0.49$) and COH ($r = 0.37$) with age also suggests a decreased function or number of the peripheral cell receptors which bind LDL and remove cholesterol from plasma. Since it is the LDL receptor binding mechanism which regulates cholesterol entrance and thereby synthesis within peripheral cells [8] any decrease in this activity will result in increased cholesterol synthesis and turnover by peripheral cells to meet their requirement of cholesterol for cell membrane synthesis. Goldstein and Brown [200] observed that as the rate of growth of cultured cells (human skin fibroblasts) declines, the number of cells receptors which bind LDL also decreases. Our results also suggest this trend.

A rise in serum and LDL cholesterol with increase in age has been reported in many studies [52-57] and our results, too, confirm this trend. The increase in plasma cholesterol was almost entirely in LDL with insignificant increases in VLDL and Connor et al. [66] have made the same observation. Our results show a significant and positive association of plasma and LDL cholesterol levels with the HMGR and COH activity in

mononuclear leucocytes. This was not surprising since it has already been demonstrated that the leucocytes of hypercholesterolemic subjects respond to incubation in a lipoprotein or lipid free medium with a greater than normal induction of HMGR [13]. Under steady conditions, in vivo, the leucocytes of these subjects do not synthesize more cholesterol, because the higher extracellular plasma LDL cholesterol in them represses the HMGR. When plasma cholesterol levels increase, less efficient mechanisms such as scavenger cells or macrophages of the reticuloendothelial system [201] become more active in degrading the increased amounts of LDL. These cells when overloaded with cholesterol esters are converted to foam cells, which are classic known components of atherosclerotic plaques.

The proportion of free to esterified cholesterol in the plasma is generally considered to be relatively constant, despite large differences in the amount of cholesterol present [72-74]. Our study showed the same trend. No studies have been noted reporting the effects of age on the chemical composition of different lipoproteins. Any modification in the chemical composition of lipoproteins can have a profound effect on exchangeability, accessibility and the chemical reactivity of lipid constituents which in turn may affect the susceptibility of peripheral tissues to lipid deposition [202]. Rothbalt et al. [203] have shown that accumulation of cholesterol esters in cell is directly related to free cholesterol to phospholipid ratio in HDL. Cholesterol esters accumulate in tissue culture cells exposed to HDL enriched in free cholesterol. Ahrens [103] and Gertler and Oppenheimer [204] suggested a relationship between lipid deposition in the arteries and increase in the cholesterol

to phospholipid ratio in plasma, and animal experiments support this hypothesis [205,206]. In our study, results show that as men increase in age, the proportion of free cholesterol to cholesterol ester in lipoproteins changes: the ratio in VLDL increases whereas both LDL and HDL show a decrease. However, none of these changes were significant in our group of men. The cholesterol to phospholipid ratio increased in plasma and all lipoproteins even though no significant changes in their phospholipid levels occurred with age. If these changes are in any way responsible for the increased cholesterol content of leucocytes in 60-70 year old men studied, it is not known.

Our results show an age related increase in plasma triglycerides, thus corroborating the findings of Carlson and Bottiger [106] and Zweers et al. [107]. The plasma triglycerides showed a significant and positive correlation with all lipoprotein triglycerides, the highest being with VLDL triglyceride (0.95). Similar results have also been reported by Olsson and Carlson [67] in his study of a random sample of 40 year old men. The significant increase in VLDL triglycerides with age suggests a decrease in efficiency of VLDL removal from plasma, and evidence for a decrease in lipoprotein lipase activity has been reported by Chlouverakis [207] and Boberg et al. [64]. Tobey et al. [108] also suggested that the age associated increase in plasma triglycerides is due to an increase in VLDL triglyceride secretion and/or a relative decrease in VLDL triglyceride removal from plasma.

Epidemiological evidence suggests that excessive dietary intake of calories or fat may be responsible for an increase in plasma cholesterol. The results of our study (Table 8), however, show a negative but not

significant correlation of calorie, protein, carbohydrate, fat and cholesterol intake with plasma cholesterol levels. The negative correlations of the calorie ($r = -0.37$) and carbohydrate intake ($r = -0.46$) with VLDL cholesterol and of total fat intake with leucocyte cholesterol levels ($r = -0.40$) were significant. We also observed a significant decrease in the calorie and fat intake with increase in age and similar trends have been observed by Munro [113], McGandy et al. [116] and in the HANES study [114]. These trends suggest that the higher nutrient intake of the 19-25 year group of men was compensated for by their more active lifestyle, causing a rapid turnover and a decrease in plasma lipids. It is also possible that for healthy subjects like the group we observed, the influence of dietary calories, fat and cholesterol on plasma cholesterol levels is overriden. The fat intake was related positively and significantly to all lipoprotein bile acids and this may be attributed to an increased turnover of bile acids with the increase in fat intake.

The results of this study indicate that the diet is not responsible for age associated increases in plasma and leucocyte cholesterol. The increase may be attributed to decreased number or function of the peripheral cell receptors which bind and remove cholesterol from plasma.

GENERAL SUMMARY AND CONCLUSIONS

The accumulation of cholesterol esters in atherosclerotic lesions and their lethal complications stress the importance of understanding cholesterol metabolism in peripheral tissues *in vivo*. Information on the control of cholesterol synthesis and utilization in peripheral tissues should increase our understanding of the mechanisms involved in the formation of atherosclerotic lesions.

This research consisted of two parts. The first part deals with the development of methods and a model for studying cholesterol and bile acid metabolism in peripheral tissues. The usefulness of leucocytes for studying cholesterol biosynthesis in man has already been reported. We demonstrated that the leucocytes can also synthesize bile acids, the end product of cholesterol catabolism. Evidence is presented for the presence of bile acids and cholesterol 7α-hydroxylase activity in leucocytes and other nonhepatic tissues. The results were also verified by chemical ionization mass spectra. That biopsy of the leucocyte would allow us to study *in vivo* responses of peripheral tissue cholesterol metabolism is suggested 1) by their ability to synthesize and catabolize cholesterol and 2) by the fact that they are a nonendocrine extrahepatic tissue and reflect LDL regulation of peripheral tissue cholesterogenesis. The second part of the study uses the leucocyte model for studying the effects of age and diet composition on cholesterol metabolism in peripheral tissues of adult men.

After the study was approved by the Human Subjects Review Committee, 29 healthy men from three age groups (19-25, 40-50 and 60-70 years) were

selected. Written participant consent of the subjects and medical approval for participation in the study were also obtained. The mean body weight of the subjects in the three groups did not differ significantly. The subjects were instructed how to record their diet for three consecutive days (2 weekdays and 1 day of weekend). These records were analyzed for energy and nutrient content by using a computer program based on the food values in the Agriculture Handbook, 456.

A 100 ml sample of fasting blood was drawn once by venipuncture. It was used for separation of mixed and mononuclear leucocytes and plasma lipoproteins. The biochemical tests performed on plasma lipoproteins and leucocytes included determination of cholesterol (total and free), phospholipids, triglycerides and bile acids. The activity of the rate limiting enzymes in cholesterol and bile acid synthesis hydroxymethyl glutaryl CoA reductase (HMGR) and cholesterol 7α-hydroxylase (COH) was measured in microsomes prepared from mononuclear leucocytes.

There were no significant differences in the number or proportion of different leucocytes in the three age groups studied. However, the mean cholesterol content of leucocytes of the oldest age group (60-70 years) was significantly higher than those of the two younger groups. The mean HMGR activity for the 40-50 year group was significantly higher than that for the youngest group, but similar to that of the older group. The COH activity for the two older age groups was higher than the mean value of the 19-25 year old group, but these differences were not significant. The plasma and LDL cholesterol values increased significantly with increase in age with a mean annual change of 1.57 and 1.29 mg/dl plasma respectively. Age had no significant affect on VLDL cholesterol. The mean HDL cholesterol

levels were similar in all three age groups. With increase in age, no significant changes were noted in the phospholipid and bile acid levels in plasma and lipoprotein. Plasma and VLDL triglycerides increased significantly with age, while LDL and HDL triglycerides were similar in all three age groups.

The diet composition data show that the mean intakes of total calories, fat and carbohydrate of 19-25 year old men differed significantly from the 40-50 year old men, but not from the 60-70 year old men. The percentage of calories derived from protein, fat or carbohydrate was similar in all the three age groups. Negative and significant effects of dietary intake of calories and carbohydrates on VLDL cholesterol and of total fat intake on leucocyte cholesterol were noted.

Our results suggest that the age related increase in plasma and LDL cholesterol may reflect a decreased number or function of the peripheral cell receptors which bind and remove cholesterol from plasma. The results also indicate that the leucocytes behave as a nonendocrine, extrahepatic tissue with a potential usefulness as a biopsy tissue for studying cholesterol synthesis and degradation in peripheral tissues. A person could serve as his/her own control for effects of various treatments such as different diets or drugs. The study of leucocytes would be particularly useful for therapeutic regimens which decrease plasma lipids and lipoproteins but increase lipid deposition in peripheral tissues.

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APPENDIX A

Statement of Information to Participants

IOWA STATE UNIVERSITY

FOOD AND NUTRITION DEPARTMENT

AMES, IOWA

TITLE OF STUDY: Effect of age and food intake on plasma lipids, lipoprotein and cholesterol metabolism in leucocytes, Nina K. Dodd, Food and Nutrition Department, Ph.D. candidate.

PURPOSE OF STUDY:

Heart attack is the great plague of modern times in economically developed countries. Most of the time a heart attack is caused by atherosclerosis (hardening of the arteries) because of accumulation of cholesterol in the walls of arteries over a period of many years.

We are interested in analyzing the cholesterol level in the blood of mean of different ages so that we may draw conclusions about how cholesterol changes with age and how food intake affects these changes. The particular part of your blood which we plan to analyze is white blood cells because they have been shown to be a good indicator of the functioning of your body.

The objectives of this research are (1) to examine the effect of age and food intake on cholesterol and (2) to investigate how cholesterol metabolism changes with age and the role this might play in atherosclerosis.

GRADUATE STUDY COMMITTEE:

Pilar A. Garcia, Major Professor (FN), Jacqueline Dupont (FN), Robert Serfass (FN), Donald Beitz (BB), and Richard Engen (VPP).

COOPERATION: Dr. Donald Hotchkiss, statistical consultant (Statistical Laboratory), Dr. L. Z. Furman, medical consultant (Student Health Service)

CRITERIA FOR PARTICIPATION IN THE STUDY:

You may volunteer to give 100 ml of blood if

1) You are a male and within one of these age groups: 18-25 yers., 40-50 yrs., 65-74 yrs. (10 needed for each group).

2) Your body weight is within the suggested desirable weight for your height (see table on back of this page and use range of weights given within parentheses).

3) You are in apparent good health. Individuals taking drugs should not volunteer to give blood as drugs change lipid metabolism.

4) You are not engaged in activities such as regular jogging, swimming or other similar exercises. Physical activities affect the lipid patterns in blood.

5) You are white (Caucasian background). Observations will be limited to this particular ethnic group because of the small number of individuals within each age group. Time and money constraints limit the group size that can be studied at any one time.

6) You are willing to record your food intake for three consecutive days (two weekdays and one day of weekend).

Procedure:

Thirty men, 10 from each of the three age groups (18-25, 40-50, and 65-74 years) will be selected. They will be taught how to record their food intake for three consecutive days (two weekdays and one day of the weekend). Their diet will then be analyzed for its nutrient content.

Shirley Sjobakken, a registered nurse at Student Health Service (SHS) will record the medical history including blood pressure and ascertain that the subjects are in good health. Then 100 ml of fasting blood will be drawn once by Linda Terry a registered medical technician at the SHS. Blood will be taken in the morning before breakfast. We will provide breakfast at the Health Service dining room.

Risks

Fasting blood (100 ml) will be taken from the vein by veni-puncture. Minimal risks, like the possible formation of a bruise like area (hematoma) and infection are involved. All blood will be drawn by a registered medical technologist at the Student Health Service under stringent aspectic conditions to minimize the risks. Some minimal pain is involved in drawing blood but this should be no greater than the discomfort experienced when blood tests are made during medical examination.

FOR YOUR PARTICIPATION, you will receive:

1) Ten dollars to cover time spent going to health service.

2) A copy of your diet analysis.

3) Results obtained from this study upon request.

4) Transportation to and from the health service if needed.

Fasting Blood Samples

TIME: Before breakfast between 7:00 and 8:00 A.M.
at University Student Health Service

By: Registered Medical Technician

a. The night before a fasting blood sample is taken:
DO NOT EAT OR DRINK ANYTHING AFTER 10:00 P.M.

b. The following morning a sample is taken:
DO NOT EAT OR DRINK ANYTHING.
We will provide breakfast at the Health Service dining room.

c. Dates when samples will be taken:
February 21, 1981. Friday
February 25, 1981. Wednesday
March 2, 1981. Monday

d. BE AT HEALTH SERVICE BY 7:00 A.M.

Food and Nutrition Department
Iowa State University
Ames, Iowa 50011

Participant Informed Consent

I, _____, have been informed verbally and in writing of the purpose, benefits, and potential hazards of the study entitled "Effect of age and food intake on plasma lipids, lipoproteins and cholesterol metabolism in leucocytes in men" by Nina Dodd.

I volunteer of my own free will to participate fully in this study. I understand that I will be given further explanation of the study and of specific procedures, if I so desire. I also understand that I may withdraw from the study at any time and that I am not waiving my legal rights. I understand that my records will be handled in a confidential manner as medical records, and that I will be given access to my records during and at the end of the study, if I so desire.

I understand that emergency treatment of any injuries that may occur as a direct result of participation in this research will be treated at the Iowa State University Student Health Services, Student Services Building, and/or referred to Mary Greeley Hospital or another physician. Compensation for treatment of any injuries that may occur as a direct result of participation in this research may or may not be paid by Iowa State University depending on the Iowa Tort Claims Act. Claims for compensation will be handled by the Iowa State University Vice President for Business and Finance.

_____
Signature

_____
Witness

_____
Date

Medical Clearance Statement

To: Personal Physician of _____
Name of volunteer

From: Nina Dodd, Ph.D. Candidate and Dr. P. A. Garcia, Professor
Food and Nutrition Department
33A MacKay Hall
Iowa State University
Ames, Iowa 50011

Date: February 6, 1981

We are studying the effects of age and food intake on plasma lipids, lipoproteins and cholesterol metabolism in leucocytes in men. The purpose and procedure to be used are described in the statement of information to participants and a copy is attached together with the Participants Informed Consent.

To participate in our program, the individual must meet the following criteria: (a) male, (b) be in one of the age groups of 18-25 years, 40-50 years, and 65-74 years, (c) body weight is within the suggested desirable weight for his height, (d) not taking drugs, (e) not doing regular jogging or swimming or any similar exercises, (f) is in apparent good health.

Your patient _____ has volunteered to
Name
participate in our program and we would appreciate your medical recommendation concerning his/her decision. Please complete the form below and return to me.

Please check:

_____ I have no reservation regarding this patient's participation in your study.

_____ I have reservations regarding this patient's participation in your study. My reservations are as follows:

a. _____

b. _____

c. _____

_____ _____
Signature of Physician Date

Directions for Food Intake Records

1. PLEASE EAT AS YOU DO ORDINARILY so that your intake will reflect your typical pattern of eating.

2. Write down everything you eat or drink for three (3) consecutive days (2 week days and 1 day of the weekend) on the Food Record form.

3. Write down your name, date of record and information about yourself on each sheet.

4. Use the attached SAMPLE FOOD RECORD as your guide.

5. Describe foods eaten as accurately as you can. Examples are:

   Meats: Ground beef patty, pan-broiled; Breaded pork chop; Fried chicken breast

   Fruits: Small, medium or large

   Fresh Canned Fruits: Water pack, juice pack, light syrup, heavy syrup

   Breads: White enriched; White unenriched; 100% Whole Sheat; Rye; Special Brands, thin or thick sliced

   Cereals: Oatmeal; Wheaties; with whole milk; with cream; with sugar, etc.

   Condensed Soups: prepared with milk or water

   Salads: Tomato-Lettuce; Apple, celery, nuts with mayonnaise; with French dressing

   Milk: Whole; 2%; skim; buttermilk

   Casseroles and Mixed Dishes: List and estimate ingredients used

   Vegetables: Raw, cooked, canned - vacuum or liquid pack

6. Estimate amounts eaten as carefully as you can and record amounts in household measures, ounces or untis as suggested below:

Bread	Slice
Meats; Cheese	Ounces (oz.) See other side
Beverages: Cereals; Canned Fruits; Cooked Vegetables	Cups (c.)
Fresh fruits	Small, medium, large. Measure on berries.
Butter; Margarine; gravy; Salad Dressings; Toppings	teaspoon (tsp.); Tablespoon (Tbsp.)
Sugar; Jellies; Jam; Sugar Syrups	teaspoon (tsp.); Tablespoon (Tbsp.)
Cake, Cookies	Measure in inches
Carbonated Beaverages, Alcoholic Beverages	Ounces (oz.)

7. Indicate time of day food is eaten as morning meal, noon meal, evening meal or in-between meals (snacks).

8. If you are taking any vitamin or mineral pills, write down the kind and how often you take them. This will not be coded, but the information can be added individually.

THE MORE ACCURATE THE RECORD, THE MORE ACCURATE THE ANALYSIS

APPENDIX B: METHODOLOGY

Selection of Subjects

Thirty men, 10 from each of three age groups (18-25, 40-50 and 65-74 yrs) were selected. None of the subjects was engaged in activities such as regular jogging, swimming or other training exercises. Their body weights were in the desirable weight range for their height. Medical approval of the subjects was obtained to ascertain that they were in good health. Because of the small number of individuals within each age group, all subjects were white (Caucasian background).

Blood Collection

A 100 ml sample of fasting blood was drawn from the vein by venipuncture by a registered medical technician at the Student Health Service, under aseptic conditions to minimize the risks. The blood samples were processed as follows: 40 ml of blood was used for counting the number and proportion of leucocytes and for plasma and lipoprotein separation, 30 ml was used for separation of mononuclear cells and 30 ml for separation of mixed leucocytes.

Counting the Total Number of Leucocytes

Forty μl of blood was diluted with 20 ml of isoton to give 1:501 blood dilution. To this 6 drops of zap isoton was added to destroy red cells and the solution was mixed well. The aperture of the tube of a Coulter counter was immersed into the diluted blood and the total number of leucocytes recorded. Three determinations were made for each sample and the average used as the total number of leucocytes.

Differential Count of Leucocytes

Enumeration of the proportion of different kinds of leucocytes was done by examining a blood smear stained with Wright's stain under a microscope [196]. A freshly made blood smear was air dried, covered completely with Wright stain for 3-4 minutes and then washed with water until a yellowish or pink film was left. This was allowed to dry and then examined under the microscope.

Isolation of Mixed Leucocytes

Mixed leucocytes were isolated by following the Young and Rodwell method [11]. Ten ml of blood drawn in the presence of Na_2EDTA was placed in a 50 ml polycarbonate centrifuge tube containing 10 ml of 3% Dextran T500 in 0.9% NaCl. The erythrocytes were allowed to clump and settle for 45 minutes at room temperature and were then further sedimented by centrifugation (25 g, 10 min.). The supernatant liquid was removed and centrifuged at 300 g for 30 minutes to sediment the leucocytes. The leucocyte pellet was resuspended in Buffer I which contains 250 mM NaCl, 5mM K_2EDTA, 5 mM dithiothreitol and 50 mM K_2HPO_4 at pH 7.5. The suspension was stored in liquid N_2.

Isolation of Mononuclear Leucocytes

Mononuclear cells were isolated by layering over Ficoll-Paque, according to the method described in the Manual of Clinical Immunology [159]. Blood drawn in the presence of heparin was diluted with an equal volume of balanced salt solution and layered over 5 ml of Ficoll-Paque a 50 ml glass tube. Blood was added in such a way that the Ficoll-Paque layer was not disturbed. This was centrifuged at 1450 for 40 minutes.

The top layer on Ficoll-Paque, which consists of mononuclear cells was removed. Monocytes were separated from lymphocytes by adherence to culture flasks.

Isolation of Granulocytes

The lower Ficoll-Paque band contains granulocytes. This layer was removed and granulocytes were isolated. Any red cells contaminating were removed by washing with cold 0.85% NH_4Cl.

Isolation of Leucocyte Microsomes

The frozen leucocyte pellet was incubated at 37°C until just thawed. The suspension was homogenized for 15 sec. in a Potter-Elvehjem tissue grinder and the homogenate was centrifuged at 8000 g for 10 min. The supernatant was removed by a Pasteur Pipette to an ultracentrifuge tube which also filled with Buffer I. This was centrifuged at 113,000 g for 2 hours. The microsomal pellet was suspended in Buffer I by homogenization.

Separation of Plasma and Lipoproteins

Blood was centrifuged at 1000 g for 10 minutes at 4°C for separation of plasma. Plasma lipoproteins were separated by ultracentrifugation [195] into 3 major fractions - very low density lipoprotein (VLDL), low density lipoprotein (LDL 1.006-1.063) and high density lipoprotein (HDL 1.063-1.21). Ultracentrifugation of plasma for 18 hours at 20,000 g allowed the VLDL fraction to be collected at the top of the tube, from where it was removed salt (KBr) was added to the remaining (LDL + HDL) fraction to raise its density to 1.063 for isolation of LDL, after VLDL was removed. This was recentrifuged at 120,000 g for 24 hours and LDL removed from the top of the

tube. HDL + HDL bottom was centrifuged at 120,000 g for 48 hours to isolate HDL, after the density of the solution was increased from 1.063 to 1.21 by addition of KBr solution.

Chemical Analyses

All determinations were done in duplicate and averaged.

Cholesterol Analyses

Total and free cholesterol (TC & FC) concentrations were determined by the method of Allain et al. [179]. Esterified cholesterol (CE) is calculated by the difference between TC and FC. A 10 μl sample of plasma and lipoprotein was used for total cholesterol and a 30 μl sample for free cholesterol. To this was added 0.5 ml of cofactor solution made in sodium phosphate buffer (pH 7) containing 612.9 μg of sodium cholate, 81.3 μg of 4 aminoantipyrine, 658 μg of crystalline phenol, 510 μg of 6000 carbowax, 0.06 unit of cholesterol oxidase, 0.01 unit of cholesterol ester hydrolase and 33.8 units of horseradish peroxidase. The mixture was incubated at 37°C for 10 minutes, cooled and read at 500 nM in a spectrophotometer. The mixture for free cholesterol does not contain any cholesterol ester hydrolase. Concentrations of unknown samples were determined from a standard curve constructed by using cholesterol standards in isopropanol. Free and total cholesterol concentration of a sample were assayed on the same day.

Phospholipid Analyses

Phospholipid (PL) was assayed using a modification of the method of Sandhu [197]. 100 μl sample of plasma or lipoprotein was extracted with

1.5 ml of isopropanol. Standards were prepared from a solution of lecithin in hexane (2 mg/ml). Sample, standard and aliquot of isopropanal as blank were evaporated to dryness in a block heater at 100°C. To the residue, 0.4 ml of chloroform and 0.1 ml of chromogenic solution (ammonium molybdate:H_2SO_4:methanol:chloroform:water) were added and contents were mixed for 5 s heated at 100°C for 1 min. After cooling, contents were mixed for 5 s and after 5 minutes 2 ml of nonane was added. The mixture was centrifuged at 2400 rpm for 3 minutes and absorbance of supernatant was recorded at 710 nM in a spectrophotometer. Phospholipid concentrations were calculated from the lecithin standard curve.

Triglyceride Analyses

Triglyceride in plasma and lipoproteins were estimated by the method of Fletcher [198] with slight modification. A 0.2 ml sample or standard (300 mg triolein/100 ml isopropanol) was extracted with 4.8 ml of isopropanol by centrifugation at 800 g for 5 minutes. To 2 ml of clear supernatant was added 0.5 ml of 1 N potassium hydroxide and the mixture was incubated at 60°C for 5 minutes. Then 0.5 ml of sodium periodate solution (.025 M in 1.0 N acetic acid) and after 10 minutes color reagent (20 ml of 2 M ammonium acetate, 40 ml of isopropanol and 0.15 ml of acetylacetone) were added. The mixture was mixed well, incubated at 60°C for 30 minutes, cooled and read at 415 nM. Triglyceride concentrations in samples is calculated from the standard curve.

Total Bile Acid Analyses

A modification of the method of Turnberg et al. [181] was used for assaying total bile acid concentration in plasma and lipoprotein samples.

To 0.1 ml of sample or standard was added 0.5 ml of 0.005 M β NAD and 0.6 ml of 0.1 M sodium pyrophosphate buffer. This was allowed to equilibrate at 25°C for 15 minutes and absorbance of the mixture was recorded at 340 nm. Then 0.1 ml of 3α hydroxysteroid dehydrogenase was added and change in absorbance was read at 340 nm after letting it sit for 40 minutes. Total bile acid concentration of unknown samples is read from the plot of change in absorbance vs. concentration.

Hydroxymethylglutaryl Coenzyme A Reductase (HMGCoA Reductase) Assay

HMGCoA reductase activity was measured in leucocyte microsomes by a slight modification of the Young and Rodwell method [11]. A 100 μl sample of microsomal suspension containing 0.75 mg of protein was added to 50 μl of substrate-cofactor solution to give final concentrations as follows: 20 nM[3-^{14}C] HMG-CoA sp act 58.6 mci/mmol (.07 μ ci per assay), 3 mM NADP, 30 MM glucose 6 phosphate and 0.3 units of glucose-6 phosphate dehydrogenase. The mixture was incubated at 37°C for 60 min. The reaction was stopped by adding 100 μl (40,000 dpm) of [5-^{3}H] mevalanol-actone as an internal standard. This was incubated for 15 minutes at 37°C to lactonize the mevalonate formed and then centrifuged (10,000 g) for 2 minutes to sediment protein. About 0.5 g of sodium sulfite was added to each tube (pH ~ 6.5) and mixture was extracted thrice with 5 ml of benzene. The benzene extract was dried and counted with 10 ml of Bray's solution in liquid scintillation counter using external standard channel ratio. The calculations were done as follows:

$$\text{pmols formed per 60 minutes} = C^{14} \text{ added} \times \frac{0.003}{\text{cpm lactone added}} \times \frac{\text{cpm in } {}^{3}H \text{ channel}}{\text{cpm in } {}^{14}C \text{ channel}}$$

Cholesterol 7α-Hydroxylase Assay

Cholesterol 7α-hydroxylase assay in leucocyte and tissue microsomes was done using Carlson et al. method [163] with following modifications.

The [4-^{14}C] cholesterol (54 ci/mol) was purified by TLC using benzene/ethyl acetate (2:3 v/v) with a standard in an adjacent channel. The plate was broken in half and standard was sprayed with 50% H_2SO_4 and heated in an oven for 1-2 min. A comparable band on the section of plate containing 4-^{14}C cholesterol was scraped and extracted with acetone. This was evaporated under N_2 to reduce volume to give approximately 2 x 10^5cpm per 150 µl. The cholesterol suspension was prepared by adding Tween 80 (0.36 mg per assay) to cholesterol in acetone, evaporating to dryness under nitrogen and resuspending residue to appropriate volume with deionized water.

Microsomal suspension (0.3 ml) in homogenizing buffer was incubated at 0°C for 10 minutes with 0.3 ml of potassium phosphate buffer (pH 7.4) containing 35 mM β mercaptoethanol, 0.15 ml of 4.5 mM $MgCl_2$ and 0.15 ml of cholesterol suspension. This is to allow equilibration of exogenous and microsomal cholesterol. Substrate cofactor solution (0.15 ml) containing 0.75 mM NADP, 3 mM glucose 6 phosphate and 0.15 units of glucose 6 phosphate dehydrogenase are then added. The mixture is incubated at 37°C for 40 minutes with shaking. Blanks containing boiled microsomal suspension were also treated in the same manner. The reaction was stopped with 2 ml of ethanol and extracted with petroleum ether, after vortexing.

The extract was dried under nitrogen, dissolved in 0.125 ml of benzene/methanol (4:1, v/v) and applied to TLC plate under nitrogen, with cholesterol and 7α-hydroxycholesterol as a standard. The plates were developed with benzene/ethyl acetate (2:3) for 40-45 minutes. Cholesterol and 7α-hydroxycholesterol were identified by spraying with 50% H_2SO_4 and these bands were removed and assayed for radioactivity with scintisol using liquid scintillation spectrometry. The calculations were done as follows:

%conversion of [4-^{14}C]-cholesterol to [4-^{14}C]-7α-hydroxycholesterol

$$= 100 \frac{(\text{cpm in 7α-hydroxycholesterol (TLC)}}{\text{cpm in cholesterol (TLC)}} \times \text{cpm cholesterol added.}$$

APPENDIX C: INDIVIDUAL DATA OF EACH SUBJECT

Table C-1. Age, weight, blood pressure and leucocyte count in men of 3 age groups

Age Group (yrs)	Subject Number	Age (yrs)	Weight (lbs)	Systolic (mm Hg)	Diastolic (mm Hg)	Leucocyte Count	Mononuclear Leucocytes (%)
	1	22	175	118	74	10897	35
	2	24	186	118	76	7130	43
	3	24	189	---	--	6300	35
	4	20	176	124	70	5955	48
19-25	5	25	194	94	56	6522	50
	6	22	143	100	68	7153	48
	7	23	199	152	84	7794	63
	8	19	156	120	80	5960	43
	9	22	164	122	58	6153	41
	10	23	176	118	66	8079	35
Mean		22	176	118	70	7194	44

Table C-1. (Continued)

Age Group (yrs)	Subject Number	Age (yrs)	Weight (lbs)	Systolic (mm H_g)	Diastolic (mm H_g)	Leucocyte Count	Mononuclear Leucocytes (%)
40-50	1	44	172	88	64	7986	35
	2	46	179	106	74	5357	55
	3	50	155	116	86	4337	46
	4	47	172	140	98	6282	40
	5	41	136	124	80	7083	24
	6	40	207	130	84	6729	40
	7	42	---	110	72	7998	29
	8	40	162	112	70	7220	39
	9	45	208	114	62	5451	54
Mean		44	174	116	77	6494	40

Table C-1. (Continued)

Age Group (yrs)	Subject Number	Age (yrs)	Weight (lbs)	Systolic (mm H_g)	Diastolic (mm H_g)	Leucocyte Count	Mononuclear Leucocytes (%)
	1	67	165	136	78	6072	40
	2	68	178	128	86	7956	29
	3	67	167	156	96	7532	31
	4	62	165	134	84	6961	36
60-70	5	63	127	138	68	6350	61
	6	65	169	152	74	8115	40
	7	62	183	134	78	7400	39
	8	61	191	120	76	7240	28
	9	62	195	148	90	13783	36
	10	63	186	120	58	6754	29
Mean		64	173	137	79	7816	38

Table C-2. Diet composition of each subject (mean of 3 days)

Age Group (yrs)	Kcals	Protein (g)	Carbohydrate (g)	Fat (g)	Saturated Fat(g) (Total)	Oleic (g)	Linoleic (g)	P/S	Cholesterol (mg)
19-25	2981	125	312	143	39.2	40.7	8.90	0.227	393.1
	2539	81	339	99	44.1	33.8	10.30	0.233	276.5
	2066	77	219	101	31.6	33.9	13.20	0.418	266.2
	3135	100	368	147	44.9	44.8	16.30	0.363	762.2
	2998	97	304	159	50.6	63.7	24.80	0.490	420.0
	3772	143	489	124	51.2	49.4	29.70	0.580	409.2
	2811	132	266	136	64.2	49.9	21.10	0.329	712.9
	2136	93	252	88	76.2	60.1	50.40	0.661	646.4
	2432	66	244	80	34.5	32.7	10.90	0.316	215.4
Mean	2763	102	310	120	48.5	45.4	20.6	0.402	455.7

Table C-2. (Continued)

Age Group (yrs)	Kcals	Protein (g)	Carbohydrate (g)	Fat (g)	Saturated Fat(g) (Total)	Oleic (g)	Linoleic (g)	P/S	Cholesterol (mg)
	2129	95	212	99	42.1	30.4	10.10	0.239	291.3
	1481	60	184	58	32.4	40.8	14.40	0.444	667.2
	2899	106	278	108	21.1	22.5	8.50	0.403	170.4
	751	26	72	41	43.8	33.4	13.90	0.317	474.6
40-50	1134	42	145	42	32.4	53.4	13.90	0.429	467.5
	2160	86	179	119	14.8	14.2	6.30	0.426	160.9
	2239	107	239	98	13.4	15.2	9.50	0.708	119.4
	2213	79	266	90	32.8	33.6	17.90	0.545	253.2
	2333	95	296	90	34.9	26.5	19.10	0.547	413.0
Mean	1927	77	208	83	29.7	30.0	13.6	0.451	335.3

Table C-2. (Continued)

Age Group (yrs)	Kcals	Protein (g)	Carbohydrate (g)	Fat (g)	Saturated Fat(g) (Total)	Oleic (g)	Linoleic (g)	P/S	Cholesterol (mg)
	2127	64	263	68	26.5	24.1	10.90	0.411	256.6
	2291	78	323	67	18.3	15.7	4.20	0.229	177.1
	2225	235	196	116	38.2	38.7	9.90	0.259	369.9
	1903	75	164	89	29.5	26.5	9.90	0.335	601.6
60-70	1991	94	237	73	75.1	72.2	22.20	0.295	837.7
	1363	65	172	48	31.9	32.3	12.10	0.379	263.4
	2193	77	357	61	29.5	30.7	25.20	0.854	556.3
	2779	119	335	103	20.3	26.7	14.50	0.714	340.0
	2650	92	246	126	47.9	53.8	23.60	0.492	548.7
	3701	156	283	192	19.3	22.3	8.96	0.464	199.6
Mean	2322	106	258	94	33.6	34.3	14.1	0.443	415.1

Table C-3. HMGCoA reductase (HMG) and cholesterol 7α-hydroxylase (COH) activity in mononulcear leucocyte

Age Group (yrs)	HMG (per mg microsomal protein)	COH	HMG (per ml blood)	COH	HMG (per 10^7 mononuclear leucocytes)	COH
	9.3	0.095	9.8	0.100	25.7	0.262
	9.9	0.101	7.6	0.077	24.7	0.251
	13.8	0.128	12.4	0.115	56.2	0.521
	11.1	0.113	9.2	0.093	32.2	0.326
19-25	9.5	0.098	8.2	0.085	25.1	0.260
	11.4	0.104	8.1	0.073	23.6	0.214
	12.4	0.132	10.5	0.112	21.4	0.227
	10.3	0.108	9.8	0.102	38.2	0.397
	7.7	0.065	7.2	0.061	28.5	0.242
	10.2	0.116	8.2	0.093	28.9	0.331
Mean	10.5	0.106	9.1	0.091	30.4	0.303

Table C-3. (Continued)

Age Group (yrs)	HMG (per mg microsomal protein)	COH (per mg microsomal protein)	HMG (per ml blood)	COH (per ml blood)	HMG (per 10^7 mononuclear leucocytes)	COH (per 10^7 mononuclear leucocytes)
40-50	11.4	0.079	9.9	0.069	35.4	0.234
	11.5	0.096	10.6	0.089	39.9	0.447
	8.7	0.092	12.4	0.131	62.1	0.522
	13.4	0.114	11.9	0.101	47.3	0.404
	16.9	0.131	16.5	0.128	97.1	0.753
	14.3	0.108	9.0	0.068	33.4	0.253
	12.7	0.116	13.4	0.122	57.8	0.525
	13.9	0.109	12.2	0.096	43.3	0.340
	15.4	0.116	13.6	0.087	46.2	0.296
Mean	13.1	0.107	12.2	0.099	51.4	0.419

Table C-3. (Continued)

Age Group (yrs)	HMG (per mg microsomal protein)	COH (per mg microsomal protein)	HMG (per ml blood)	COH (per ml blood)	HMG (per 10^7 mononuclear leucocytes)	COH (per 10^7 mononuclear leucocytes)
	11.9	0.151	8.7	0.110	35.8	0.453
	17.3	0.156	14.0	0.126	60.7	0.547
	15.6	0.148	13.7	0.130	58.7	0.557
	12.1	0.112	12.6	0.116	50.3	0.464
60-70	9.6	0.091	10.3	0.097	26.6	0.252
	18.8	0.159	14.3	0.120	44.1	0.372
	13.9	0.142	11.9	0.121	41.2	0.419
	9.7	0.058	9.9	0.059	35.9	0.216
	21.4	0.165	16.9	0.130	34.1	0.262
	13.6	0.098	12.8	0.092	65.3	0.472
Mean	14.4	0.128	12.5	0.110	45.2	0.401

Table C-4. Total cholesterol (TC) and free cholesterol (FC) levels in plasma, lipoproteins and leucocytes

Age Group (yrs)	Plasma TC	Plasma FC	VLDL TC	VLDL FC	LDL TC	LDL FC	HDL TC	HDL FC	Leucocyte TC	Leucocyte FC
	mg/dl plasma								μg per 10^8 cells	
	190	47.4	19	4.9	120	33.6	47	10.8	139	112
	193	45.4	19	4.2	112	24.5	52	11.6	142	110
	241	62.7	24	5.7	118	26.4	49	10.6	44	34
	191	49.7	20	5.1	121	31.9	46	11.8	121	106
19-25	208	55.2	16	3.7	125	25.4	61	12.5	98	82
	195	50.3	17	3.9	123	29.8	47	9.5	45	34
	216	60.5	19	3.9	149	32.1	49	11.3	65	38
	202	56.6	24	5.8	127	32.3	39	9.3	111	82
	182	50.9	25	5.2	111	28.6	42	9.9	139	112
	209	54.2	21	4.9	166	39.7	44	11.7	102	72
Mean	204	53	25	4.7	127	30.4	48	10.9	101	78

Table C-4. (Continued)

Age Group (yrs)	Plasma TC	Plasma FC	VLDL TC	VLDL FC	LDL TC	LDL FC	HDL TC	HDL FC	Leucocyte TC	Leucocyte FC
	mg/dl plasma								μg per 10^8 cells	
40-50	215	53.7	25	5.5	138	32.8	47	13.9	162	124
	213	55.1	21	4.3	141	23.9	48	12.6	172	128
	218	61.1	11	3.2	149	22.6	51	11.9	55	41
	232	64.8	22	4.6	160	31.4	40	10.3	78	64
	308	89.3	27	5.7	130	35.2	45	12.3	137	101
	254	71.2	34	8.4	179	38.8	44	9.2	117	83
	216	58.3	24	4.1	129	22.8	46	14.6	88	70
	194	46.3	14	3.3	132	29.2	46	7.4	69	48
	239	56.4	28	8.7	171	40.2	53	12.8	73	52
Mean	232	62	21	5.3	145	30.7	47	11.7	106	79

Table C-4. (Continued)

Age Group (yrs)	Plasma TC	Plasma FC	VLDL TC	VLDL FC	LDL TC	LDL FC	HDL TC	HDL FC	Leucocyte TC	Leucocyte FC
	mg/dl plasma								μg per 10^8 cells	
	291	84.4	24	6.3	204	46.8	49	12.8	114	90
	272	73.4	13	3.9	208	42.7	44	9.5	143	93
	257	66.8	21	4.5	178	40.2	49	10.4	66	52
	279	72.7	22	5.2	199	34.1	40	8.2	128	107
60-70	226	65.5	31	10.1	157	33.2	55	12.9	71	55
	317	85.6	37	6.4	209	39.6	43	8.9	193	141
	295	75.4	20	6.4	211	41.3	64	12.3	360	249
	286	69.1	21	6.7	208	41.1	38	8.7	114	83
	309	71.1	23	7.7	221	46.8	49	8.9	113	88
	294	72.7	28	8.2	213	45.2	39	7.7	53	42
Mean	283	74	25	6.5	193	41.4	46	9.8	135	100

Table C-5. Phospholipid (PL) and triglyceride (TG) and bile acid levels in plasma and lipoproteins

Age Group (yrs)	Plasma PL	HDL PL	LDL PL	VLDL PL	Plasma TG	VLDL TG	LDL TG	HDL TG	Plasma BA	HDL BA	LDL BA	VLDL BA
	mg/dl plasma								μ Moles/dl plasma			
	266	63	161	40	98	56	29	10	0.895	0.294	0.42	0.161
	134	51	56	26	102	61	26	15	0.680	0.210	0.30	0.114
	276	64	164	43	94	58	22	11	0.340	0.105	0.15	0.084
	183	47	107	28	84	48	23	12	1.150	0.243	0.69	0.119
19-25	139	49	64	25	82	49	26	11	0.910	0.206	0.59	0.098
	277	62	170	43	79	43	22	13	0.920	0.221	0.59	0.094
	289	65	184	37	104	63	23	17	0.770	0.263	0.39	0.103
	160	38	73	29	90	51	23	14	0.410	0.210	0.30	0.098
	139	43	59	29	114	64	30	18	0.760	0.221	0.43	0.094
	234	58	181	32	88	51	24	10	0.660	0.185	0.37	0.087
Mean	210	54	122	33	93	54	24	13	0.749	0.216	0.423	0.105

Table C-5. (Continued)

Age Group (yrs)	Plasma PL	HDL PL	LDL PL	VLDL PL	Plasma TG	VLDL TG	LDL TG	HDL TG	Plasma BA	HDL BA	LDL BA	VLDL BA
	mg/dl plasma								μ Moles/dl plasma			
40-50	216	52	135	28	89	52	27	9	0.820	0.178	0.51	0.108
	179	54	96	28	96	51	30	12	0.760	0.168	0.48	0.099
	234	63	135	34	118	67	31	18	0.920	0.206	0.59	0.107
	220	45	141	32	114	69	28	12	0.440	0.146	0.21	0.066
	257	54	162	34	127	78	31	14	1.410	0.252	0.36	0.123
	197	58	102	35	94	62	19	11	0.720	0.244	0.35	0.107
	183	43	104	28	106	59	29	17	0.740	0.238	0.34	0.138
	154	50	82	21	117	74	24	13	0.560	0.174	0.25	0.096
	231	52	109	29	106	68	22	12	0.540	0.182	0.26	0.092
Mean	208	52	118	30	107	64	27	13	0.767	0.198	0.402	0.104

Table C-5. (Continued)

Age Group (yrs)	Plasma PL	HDL PL	LDL PL	VLDL PL	Plasma TG	VLDL TG	LDL TG	HDL TG	Plasma BA	HDL BA	LDL BA	VLDL BA
	mg/dl plasma								μ Moles/dl plasma			
	187	52	101	31	131	86	29	12	0.720	0.238	0.34	0.117
	130	44	66	21	112	72	25	14	0.96	0.210	0.61	0.102
	313	59	221	28	121	82	27	11	0.62	0.210	0.30	0.096
	200	39	130	27	138	95	24	17	0.35	0.161	0.23	0.094
60-70	224	54	147	23	88	58	17	10	0.53	0.147	0.26	0.092
	299	52	208	39	148	97	31	19	0.49	0.161	0.23	0.088
	176	54	94	26	109	75	21	12	0.41	0.133	0.19	0.069
	104	41	68	26	118	79	27	11	0.64	0.182	0.36	0.096
	254	55	156	40	137	101	22	13	0.64	0.224	0.31	0.096
	261	50	205	42	126	92	23	13	0.73	0.226	0.34	0.124
Mean	215	50	140	30	122	84	25	13	0.609	0.189	0.317	0.097

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