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Carol Cochran for the degree of Master of Science

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Title: The effect of lecithin supplementation on human blood lipids, coagulability, and cholesterol esterification.

Abstract approved:

Elisabeth S. Yearick

A study was made of the effects of lecithin supplementation in order to assess possible changes in plasma factors related to atherosclerosis. Subjects were 30 middle-aged males, mean age 49; the group was fairly sedentary, but had low blood cholesterol (mean, 202 mg/dl), and was on the average only 5% above their desirable weight for height. Their recorded, free-living dietary intakes were low in calories (mean, 2209), fat (mean, 90 g), and cholesterol (mean, 311 mg), but the majority had adequate intakes of required nutrients. The treatment given was 10.8 g soya lecithin per day, taken in capsular form with meals; the experimental period lasted six weeks.

A 19% increase in plasma triglycerides was noted after lecithin supplementation. No changes in plasma cholesterol, phosphatidyl choline, or lysophosphatidyl choline were seen. The percent of lipoproteins as LDL decreased slightly; HDL and VLDL did not change significantly.

Lecithin:cholesterol acyltransferase (LCAT) activity increased by 23%, although no changes were seen in levels of its substrates, phosphatidyl choline and free cholesterol, or its products, lysophosphatidyl choline and cholesterol ester. Outlets must exist for the

products formed by the reaction so that plasma concentrations remain in a steady state. The possible transfer of cholesterol ester formed by LCAT from HDL to VLDL is discussed.

The effects of lecithin supplementation on the thrombus-forming tendency of the blood was measured by the extent of ADP-induced aggregation and by recalcified plasma clotting times. No changes were observed in platelet aggregation, nor was it significantly correlated with plasma lipids. However, clotting time was shortened after the experimental period. This heightened coagulability is not a favorable response to lecithin supplementation for those individuals who are at risk of cardiovascular disease.

THE EFFECT OF LECITHIN SUPPLEMENTATION ON HUMAN BLOOD LIPIDS, COAGULABILITY, AND CHOLESTEROL ESTERIFICATION

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Carol Marie Cochran

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Professor of Foods and Nutrition
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Head of Foods and Nutrition Department

Dean of Graduate School

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INTRODUCTION[1]

The possible role of lecithin as a preventative or cure for atherosclerosis has been under consideration for at least twenty years, and recent research has indicated some links between lecithin and blood factors related to atherosclerosis. Lecithin is currently advocated as a self-help treatment for atherosclerosis (Davis, 1965; Williams, 1971; Bricklin, 1977), although such treatment is not often prescribed by members of the medical establishment. While current knowledge about lecithin does not supply enough evidence to justify claiming a role for lecithin in allaying atherosclerotic disease, this molecule is known to hold a key position in lipoprotein metabolism, particularly by its participation in the lecithin:cholesterol acyltransferase reaction, and is important in the blood clotting mechanism as well.

The research reported here was undertaken to elucidate the effects of oral lecithin on plasma factors related to atherosclerosis. Thirty middle-aged normolipidemic males ingested 10.8 g of soya lecithin daily with meals for six weeks. Their plasma lipids, coagulability, and cholesterol esterification were analyzed. The findings provide a basis for assessing the value of lecithin supplementation in the prevention of selected risk factors associated with cardiovascular disease. Perhaps more importantly, the results shed further light on the interrelationships among plasma lipids in human metabolism.

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[1] Abbreviations used in this paper: TC, total cholesterol; FC, free cholesterol; CE, cholesterol ester; PC, phosphatidyl choline; LPC, lysophosphatidyl choline; LCAT, lecithin:cholesterol acyltransferase.

REVIEW OF LITERATURE

Atherogenesis

Lecithin has been implicated as an anti-atherogenic agent, and this research was designed to observe the effects of lecithin on factors relating to atherosclerosis, such as the concentrations and metabolic pathways of plasma lipids and the tendency for thrombus formation to occur. Therefore, it is important to see how these factors fit as pieces in the puzzle of atherogenesis. In spite of recognition of many of the risk factors of atherosclerosis, its pathogenesis is far from obvious. One explanation which ties together the components of hyperlipidemia, thrombosis, and growth of the atherosclerotic lesion, is the "response to injury" theory (Ross and Glomset, 1976). These authors propose that factors such as hyperlipidemia and the increased shear stress associated with hypertension injure the arterial endothelium. Focal desquamation exposes underlying connective tissue to platelets which adhere, are induced to aggregate by the presence of collagen, and release granules. The granules contain serotonin, ADP, lysosomal enzymes, and an unknown dialyzable factor which promotes migration of smooth muscle cells from the media of the arterial wall to the intima where they proliferate. The smooth muscle cells become laden with lipid and form an extracellular matrix upon which lipid deposition occurs. The net lesion is a fibrous cap protruding into the lumen and an underlying deep deposit of free lipid. This lesion regresses unless repeated injury at the site prolongs it. A complicated lesion with calcification of the fibrous plaque may form and is associated with occlusive disease.

Platelet Aggregation

Platelet aggregation and the release of granules play a key role in the inception of atherosclerosis, according to the "response to injury" theory. The mechanisms of these responses have been studied and described in detail (Born, 1962; Marcus, 1969; Mustard and Packham, 1970; Mustard et al., 1975). Among the agents which induce platelet aggregation are thrombin, collagen, ADP, serotonin, epi-

nephrine, snake venom, long-chain saturated fatty acids, and antigen-antibody complexes. These substances cause the platelets to release ADP which is the aggregating agent; its action may be due to a reduction of the negative charge on platelet membranes, which permits adherence of cells to each other. Bolton et al. (1967) reported that patients with ischemic heart disease were abnormally sensitive to ADP. These investigators found that the causative agent was present in the low density lipoprotein, and indirectly showed it to be lysophosphatidyl choline. They postulated that plasma phospholipids altered the ability of platelet surfaces to change charge density in the presence of ADP. In vitro studies indicate that high levels of lysophosphatidyl choline produce irreversible platelet aggregation, whereas both lecithin and linolenate inhibit the aggregation initiated by saturated fatty acids (Kerr et al., 1965). However, an opposite effect of lysolecithin was seen by Besterman and Gillett (1971, 1973) who showed that 1) lysolecithin reduced the irreversible aggregation of platelets induced by ADP or epinephrine, and 2) intravenous heparin increased lysolecithin formation and reduced the irreversible aggregation initiated by collagen or epinephrine. Gillett and Besterman (1975) also found that plasma and platelet lysolecithin were lower in subjects with ischemic heart disease than in normal subjects.

Nishizawa et al. (1969) reported an inhibition of platelet aggregation in pigs and dogs fed phosphatidyl serine. Studies in rats have also shown an effect of diet on platelet aggregation (Renaud et al., 1970); the susceptibility to aggregation was higher when dietary fat was saturated than when it was unsaturated. In a study of platelet aggregation in guinea pigs, Cusack (1978) found greater sensitivity to ADP-induced aggregation in cholesterol-fed animals but reduced sensitivity when lecithin was fed with the cholesterol. Even when the diet contained no cholesterol, lecithin feeding induced a lowered sensitivity of platelets to ADP-induced aggregation. Of special interest in Cusack's study was the histological examination of the aortas of the animals to detect plaque formation. Cholesterol-fed animals showed the greatest number of plaques; animals fed cholesterol plus lecithin had higher total body

cholesterol but fewer plaques in the aorta. Cusack suggested that the presence of plaques and the resultant adhesion of platelets to the damaged vessel wall sensitizes the platelets to aggregate more readily in the presence of inducing agents, signaling an increase in the thrombus-forming tendency of the blood.

Up to now, no studies have been done with human subjects which have measured the effect of dietary phospholipid on platelet function. However, platelet aggregation has been shown to be correlated with plasma lipids in humans. According to Carvalho et al. (1974), patients with type II hyperlipoproteinemia had much greater sensitivity to aggregating agents and higher release of ADP by platelets than did normal subjects. Type IV patients, on the other hand, had normal sensitivity and release of nucleotides. This study provides evidence that, in type II disease, altered platelet function is associated with thrombotic complications and accelerated atherogenesis as suggested by the "response to injury" model. Miller and Nordøy (1977) were unable to show an acute effect of plasma lipoproteins (which are carriers of phospholipids) on platelet sensitivity to aggregating agents. Their experiments were on plasma incubated with added lipoprotein for 10 to 30 minutes, which was sufficient to allow the lipoproteins to bind to the platelet membranes. Thus, although epidemiologic studies show that the risk of atherosclerosis is augmented by prolonged elevations of the plasma lipoproteins, acute changes in lipoproteins are not the cause of altered platelet function.

Plasma Lipids

Epidemiologic surveys have indicated that elevated plasma cholesterol and triglycerides are associated with greater risk of cardiovascular disease and the highest morbidity is found in patients with multiple lipid abnormalities (Kannel et al., 1967). Therefore, interest in lecithin supplementation was stimulated by the report that 36 g of soya lecithin per day, along with a low fat diet for three months, markedly reduced the plasma cholesterol concentrations in hypercholesterolemic patients (Morrison, 1958). The soya lecithin used in this study was a mixture of phospholipids, of which 30% was pure lecithin or phosphatidyl choline. Later investigators, using

polyunsaturated phosphatidyl choline, attempted to confirm Morrison's findings, with mixed success. A study of 114 atherosclerotic patients, half of whom received 0.6 to 1.8 g of phosphatidyl choline for two years, revealed no differences in plasma cholesterol or phospholipids between the test and control groups (Enticknap, 1962). Likewise, ter Welle et al., (1974) noted no changes in plasma cholesterol or phospholipids in hypercholesterolemic subjects given 0.6 to 2.4 g of phosphatidyl choline (PC) daily. On the other hand, Svanberg et al. (1974) reported a 16% decrease in serum triglycerides and an increase in high density lipoproteins of five hypertriglyceridemic males, after five weeks of daily treatment with 1.7 g lecithin linoleate.

Holden (1976) studied the effects of lecithin supplementation on plasma lipids of normolipidemic males. Daily supplements of 7.2 g soya lecithin (2.7 g PC) for eight weeks produced no changes other than a 2% increase in the percent of cholesterol ester. This increase was attributed to an increased activity of the esterifying enzyme, lecithin:cholesterol acyltransferase (LCAT).

Lipoproteins

One well-known risk factor in vascular disease is a high level of low density lipoprotein cholesterol, while high density lipoprotein cholesterol is thought to be protective (Gordon et al., 1977). It has been postulated that the high density lipoproteins (HDL) may remove cholesterol from tissues and transport it to the liver for excretion in bile (Glomset, 1970). The low density lipoproteins (LDL) may be the source of the lipid in the atherosclerotic plaque. Lecithin may act in a protective manner by reproportioning the lipoprotein cholesterol so that more is in HDL but less is in LDL. Childs et al. (1977) found that normolipidemic subjects, given 36 g soya lecithin daily for three weeks, showed lowered LDL cholesterol and, in women, increased concentrations of HDL cholesterol. The polyunsaturated fatty acid (PUFA) content of lecithin could account for the effect on the LDL cholesterol since the same effect was found when corn oil, containing equivalent PUFA, was fed instead of lecithin. However, corn oil did not raise the HDL cholesterol. In the study of Svanberg et al. (1974)

cited earlier, oral lecithin linoleate produced an increase in HDL cholesterol in hypertriglyceridemic males as well as a decrease in plasma triglycerides. The effect on HDL was presumed to be due to activation of the LCAT reaction by better substrates, namely HDL-bound polyunsaturated phosphatidyl choline.

A recent study with chimpanzees (Rosseneu et al., 1979) showed that the PUFA content of dietary lecithin was a major factor in alteration of lipoproteins. After one month of a diet including 37.6 g of added highly polyunsaturated phospholipid, an increase in cholesterol ester and lysophosphatidyl choline was seen in the very low density lipoprotein (VLDL) and the HDL_3 fractions. The HDL_3 is a subfraction of HDL which carries large amounts of PC and free cholesterol (FC), which are substrates for the LCAT enzyme. Such a change in composition would produce a particle with a denser core and a more fluid surface with, perhaps, an improved ability to carry cholesterol away from cells, thus protecting against the development of atherosclerosis. When 26.6 g of highly saturated PC was substituted for the polyunsaturated phospholipid in the chimpanzee diet, the LDL, VLDL, and triglyceride (TG) levels were all higher and the cholesterol esters were more saturated. All of these effects are thought to enhance atherosclerosis.

Wallentin (1977a) found no change in the plasma lipoproteins or LCAT activity in seven healthy males given 2.25 g of oral polyunsaturated lecithin daily for one to four weeks. However, intravenous injection of phosphatidyl choline increased the HDL concentrations and the LCAT activity. HDL cholesterol and TG concentrations were unchanged. He concluded that an excess of plasma lecithin altered the lipoproteins by stimulating LCAT activity.

Lecithin:cholesterol Acyl Transferase

The LCAT reaction is a transesterification of a fatty acid from lecithin to free cholesterol forming the products lysolecithin (LPC) and a cholesterol ester (CE). LCAT usually transfers the unsaturated fatty acid from position 2 of PC, but fatty acids from position 1 also form CE, depending on their degree of unsaturation (Assman et

al., 1978). LCAT is thought to be the major source of CE in plasma (Glomset et al., 1966; Glomset, 1970) and to occur on the surface of HDL where the substrates PC and FC are located (Norum, 1973). Glomset (1972) postulated that the substrates FC and PC are transferred from VLDL and LDL to HDL, and that HDL transfers the LCAT-produced CE to VLDL. Rose and Juliano (1979) showed a significant transfer of CE from HDL_3 and very high density lipoprotein to VLDL in vitro. It is also possible that HDL gains FC from plasma membranes. The function of LCAT might then be to convert excess cholesterol synthesized in the tissues to a transport form, cholesterol ester, which can be taken by the lipoproteins to the liver for catabolism. Another role of LCAT may be to supply LPC to tissues for resynthesis of PC for use in membranes (Wille et al., 1978).

LCAT has been shown to be activated by apo A-I, an apoprotein present on the HDL (Fielding et al., 1972; Soutar et al., 1975) but high levels of apo A-I inhibit the enzyme (Albers, 1978; Soutar et al., 1975; Fielding et al., 1972). Apparently, the LCAT activity is primarily on the HDL_3 fraction where it is stimulated by apoprotein A-I and by the high substrate concentrations on the lipoprotein surface. Apo C-I, present primarily on VLDL but also on HDL, is another activator of LCAT (Soutar et al., 1975; Kostner, 1978; Albers, 1978).

Cramp and Tickner (1978) and Leiss et al. (1978) have proposed that VLDL is the preferred substrate for LCAT action. The former group postulated that the esterification of surface cholesterol by LCAT renders the lipoprotein surface less stable and more easily penetrated by lipoprotein lipase. TG is diminished by the lipase and the VLDL shrinks, while the HDL acts as a CE acceptor. Leiss et al. argue in a similar vein that as lipoprotein lipase removes VLDL-TG, the shrinking VLDL is enriched in surface cholesterol and lecithin. The increase in these LCAT substrates stimulates LCAT activity. However, it is likely that the main locus of LCAT action is on HDL not VLDL. HDL is a good source of LCAT substrates and the activator apo A-I. The CE produced by LCAT on HDL can be extensively transferred to VLDL (Rose and Juliano, 1979). The activation of LCAT by VLDL apo C-I can occur during interactions between lipopro-

teins.

Evidence is accumulating that the cholesterol exterification process and the metabolism of triglycerides are integrated in some as-yet-indifinite way. Nestel (1970) found a correlation between TG and CE turnover. In studies with hyperlipidemic subjects, LCAT activity was positively correlated with TG concentration in plasma (Wallentin, 1978) and with VLDL concentrations (Wallentin, 1977b). Wallentin and Vikrot (1976) showed a stimulation of molar LCAT rates by fat ingestion by normal subjects; the maximal increase in LCAT correlated positively with the maximal increase in TG. LCAT activity is thought to be increased by excess plasma PC which can arise from chylomicron catabolism following absorption. There is also greater synthesis and output of the enzyme by the liver when the plasma triglyceride secretion is higher (Nordby and Norum, 1978). Kostner (1978) reported that LCAT activity peaked at the point when plasma TG was highest. Rose and Juliano (1979) also reported an increase in molar LCAT rates after a high-fat test meal. LCAT, by esterifying cholesterol and indirectly aiding in the breakdown of TG-rich VLDL particles, may act as a central link in the metabolism of cholesterol and triglycerides.

The theory that LCAT plays an anti-atherogenic role in the removal of cholesterol from peripheral tissues needs reconsideration in light of the report that the liver selectively extracts and utilizes FC rather than CE from the HDL for the formation of bile (Schwartz et al., 1978). It is possible that the HDL selectively transports FC from the tissues to the liver and that CE of the HDL is not active in the disposal of cholesterol. Although the extraction of cholesterol from HDL was reported to be much higher than from LDL, no report was made of extraction of cholesterol from VLDL. If CE from HDL is transferred to VLDL, the extraction of VLDL-CE by the liver may be a plausible route for cholesterol removal. Although it is agreed that LCAT is the primary source of plasma CE, the significance of the generated CE is still unrevealed.

In spite of uncertainty about the exact role of LCAT in lipid metabolism, the LCAT activity has been correlated with risk factors

for cardiovascular disease. Wallentin (1977b) reported that molar LCAT rates were higher than normal in all types of hyperlipoproteinemia, and highest in types IV and V. LCAT was positively correlated with VLDL concentration, body mass, and excess body mass (Wallentin et al., 1978). A low fat diet with a high polyunsaturated/saturated fatty acid ratio did not alter the molar or fractional LCAT rates of type IIa subjects, but bile-acid binding treatment in addition to diet therapy increased the fractional LCAT rate by 30% with no change in moler LCAT rate (Wallentin, 1978).

Holden (1976) found that the LCAT reaction may be stimulated by supplemental dietary lecithin. When 12 middle-aged men ingested 7.2 g soya lecithin daily for eight weeks, the LCAT activity increased by 11%.

Absorption of Lecithin

Dietary lecithin is hydrolyzed by pancreatic phospholipase in the small intestine, forming lysolecithin and releasing a fatty acid. The LPC is rapidly absorbed and reacylated to PC in the mucosal cell (Nilsson and Borgstrom, 1967; Rodgers et al., 1975). Recent in vitro studies of the rat small intestine have shown that luminal lecithin decreases fat and cholesterol uptake by mucosal cells (Rampone, 1973; Rampone and Long, 1977; Saunders et al., 1976; Rodgers and O'Connor, 1975). It is proposed that lecithin swells the size of the lipid micelles and slows their diffusion across the unstirred water layer overlying the mucosa, thereby decreasing delivery from micelle to the absorptive cell. However, luminal PC of either dietary or biliary origin is converted to LPC, except in cases of pancreatic insufficiency or, conceivably, overloading of the phospholipase enzyme. LPC is an amphiphile which does not swell the micelle. The same studies showed that it does not inhibit fat uptake. Since PC is hydrolyzed to LPC in the intestine, supplemental dietary lecithin probably does not reduce lipid absorption nor exert a hypolipidemic effect in this way. It is worthy of note that a synthetic diether phosphatidyl choline which resists hydrolysis by pancreatic phospholipase inhibits cholesterol and fatty acid uptake by the mucosal cells in vitro and

in vivo and may be of use as a therapeutic hypocholesterolemic agent (Rodgers and O'Connor, 1975; Rodgers et al., 1977).

O'Doherty et el. (1973) showed that biliary or dietary lecithin is needed for transport of triglyceride from the mucosal cells into chylomicrons by providing a surfactant and supporting protein biosynthesis. These results were not confirmed in vivo by another group (Tso and Simmonds, 1977). It must be considered here that the intestinal cell is capable of de novo synthesis of lecithin from diacylglycerol and choline via CDP-choline, but that the pathway from LPC acylation is preferred (Mansbach, 1977). Thus, even in bile fistula animals, where biliary PC is not present and absorbed, chylomicron PC can be synthesized de novo if choline is supplied in the diet. This pathway is diminished when LPC or PC are present in the lumen. While luminal choline or phosphatidyl choline is important in the absorption of triglycerides, adequate PC is normally supplied in the bile and by de novo synthesis. About 95% of dietary fat is absorbed under the usual conditions. An increased intake of PC is not likely to increase this fat absorption.

PROCEDURE

Design of Experiment

Subjects

Middle-aged male volunteers were recruited from among university personnel. An announcement of the study appearing in the weekly Newsletter drew 30 applicants, 13 of whom had participated in a previous lecithin study. All applicants answered a short questionnaire concerning their height, weight, smoking habits, daily physical activity, and health status. Two men who had had heart attacks and one who was a diabetic were asked to obtain their physicians' approval before participating in the study.

Treatment

The sole treatment was the administration of 10.8 g oral lecithin per day for a period of six weeks. Three capsules[2], each containing 1.2 g soya lecithin in soybean oil, were taken three times daily with meals. The subjects agreed to maintain their typical dietary and exercise patterns during the experimental period. As a check on dietary intake, each participant was asked to complete a two-day dietary record at the beginning and again at the end of the study. The subjects further agreed to have a licensed medical technologist draw two venous blood samples before, and at the end of the lecithin supplementation period. The experimental plan was approved by the Human Subjects Committee of the university, in compliance with the DHEW guidelines (DHEW, 1971).

Methods

Body Weight and Activity Level

The relative weight of each subject was computed as the ratio of his weight to the desirable weight for height, as reported in the Metropolitan Life Insurance Co. tables (1959). Assignment of subjects

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[2]Natural Needs Soya Lecithin capsules, Western Wholesale Co., Portland, OR.

to activity levels was based on information they supplied on the questionnaire regarding the intensity and duration of physical exercise associated with their daily occupation and with their extracurricular activities. Activity levels were scaled from one to four, according to the type of occupation: 1) sedentary, 2) light activity, 3) moderate activity, and 4) strenuous activity (Bogert et al., 1973). In addition, if a subject exercised strenuously for 45 minutes or more, at least three times weekly, his rating was increased by one level.

Dietary Analysis[3]

Foods on the two-day diet records were coded for computation of kilocalories, protein, fat (saturated, monounsaturated, and linoleic acids), carbohydrate, iron, calcium, vitamin A, thiamin, riboflavin, niacin, and ascorbic acid. The nutrient computations were based on a data bank compiled in Home and Garden Bulletin 72 (U.S.D.A., 1971). The dietary cholesterol was computed from data compiled by Feeley et al. (1972). The nutrient values are reported as the average of each subject's intake on the two days. Daily nutrient intakes were evaluated on the basis of the recommended dietary allowances for adult men, aged 23 to 50 (Food and Nutrition Board, 1974).

Blood Collection

Twenty milliliters of blood were drawn, at fasting, on two nonconsecutive days before lecithin supplementation, and again after the six-week experimental period. At each drawing, the blood was collected into one ten-ml Vacutainer containing 14 mg EDTA and one ten-ml siliconized Vacutainer to which 3.1% sodium citrate solution was added to make up 10% of the final volume. The EDTA-anticoagulated blood was refrigerated immediately, centrifuged, and the plasma was separated. One milliliter of plasma was refrigerated for lipoprotein analyses; the remainder was stored at -12°C for lipid analyses. The citrated blood was held at room temperature, centrifuged at 500 G for ten minutes, and the platelet-rich plasma was removed. The remainder was

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[3]Dietary analyses were done by Dr. E. Yearick.

centrifuged at 1500 G for 30 minutes to obtain platelet-poor plasma. The fresh plasma fractions were tested within several hours for platelet aggregation and clotting times.

Except for the lipoprotein separations and the phospholipid analyses, all biochemical measurements were performed on four samples from each subject.

Platelet Aggregation

Platelet aggregation was measured by the method of Born (1962). Platelet-rich plasma (PRP) is treated with ADP which induces aggregation. The plasma loses its turbidity as the aggregates form from platelets and the resultant change in absorption of infra-red light is measured photometrically. A Chrono-log Model 330 Aggregometer[4] was used. This is a recording photometer, constructed so that the cuvette contents are maintained at 37°C and agitated by a magnetic stirrer. The maximum deflection was set at 1 on the recorder with platelet-poor plasma (PPP) as a baseline. Then the cuvette with 0.4 ml PRP was introduced and the PRP baseline was adjusted to 9. After one minute to assure a steady baseline, 25 ul of ADP solution were added to produce a final concentration of 1.2×10^{-5}M and the change in density was recorded for five minutes. Each PRP sample was run in duplicate. Working solutions of ADP in 0.45% imidazole-buffered saline were prepared daily from a frozen stock solution. Platelet aggregation is expressed as percent of maximum deflection from the PRP baseline.

Recalcified Plasma Clotting Time

The method for recalcified plasma clotting time was modified from Davidsohn and Wells (1962). One hundred mucroliters of platelet-rich plasma was pipetted into each of four 7 x 50 mm glass tubes containing 100 ul of normal saline solution. The tubes were incubated in a 37°C water bath. Two hundred microliters of 0.02 M $CaCl_2$ were added to each tube and mixed to a count of ten. Every 15 seconds following the $CaCl_2$ addition the tube was tilted to check for formation of a fibrin clot. The clotting time was recorded with a stop watch.

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[4]Chrono-log Corp., Havertown, PA.

Lecithin:cholesterol Acyltransferase Activity

The lecithin:cholesterol acyltransferase activity was measured by a modification of the method of Stokke and Norum (1971). In this method the substrates of the enzymatic reaction are endogenous lipoproteins which have come to isotopic equilibrium with added ^{14}C-labelled cholesterol during a four hour incubation period. In this modification, the cholesterol ester end product is separated from the free cholesterol substrate by differential elution through a column. The relative amounts of labelled FC and CE are established by scintillation counting and the enzyme activity is given as percent ester formed (CE counts x 100/ CE counts + FC counts).

One hundred microliters of thawed EDTA plasma were pipetted into each of three tubes, one blank and two replicates, containing 20 ul of 1.4 mM Ellman reagent (5, 5'-dithiobis (2-nitrobenzoic acid)) and 30 ul of an albumin-stabilized emulsion of 4-^{14}C-cholesterol (New England Nuclear 0.01 mCi/ml). The tubes were incubated with shaking for four hours at 37^{o}C to allow isotope equilibration. The enzyme was reactivated with 0.1 M mercaptoethanol and the tubes were incubated for one additional hour. The reaction was stopped and the lipids were extracted by the addition of 2.0 ml of isopropanol with prompt mixing, followed by centrifugation for 30 minutes. One hundred microliters of the supernatant were pipetted into counting vials for recovery determination; 1.00 ml of supernatant was pipetted into centrifuge tubes and dried under nitrogen for column separation.

Separation of CE and FC was performed on silicic acid-packed glass columns (10 mm OD x 70 mm with a 20 ml reservoir). The columns were filled by a slurry of silicic acid (100-200 mesh Biosil A)[5] in hexane. The dried sample supernatant was redissolved in hexane and added to the column. CE was eluted by addition of 10 ml of 2.5% ether followed by 5 ml of 5% ether in hexane; the eluate was collected in a counting vial. A new vial was placed under the column and FC was eluted with 15 ml acetone. The solvents in the vials were evaporated and 10 ml of counting fluid (one L Triton X-100/two L toluene/16.5 g

[5]Bio-Rad Laboratories, Richmond, CA.

PPO/0.5 g POPOP) were added to each vial. The samples were counted in a Beckman LS 3133 P liquid scintillation counter and esterification calculated as above.

Plasma Cholesterol and Triglycerides[6]

Isopropanol extracts of the plasmas were prepared for assay of total cholesterol, cholesterol ester, and triglyceride concentration.

Total cholesterol was determined by the acid ferric chloride color reaction (Block et al., 1966), adapted for use with the Auto-Analyzer[7]. Cholesterol ester was analyzed similarly after chromatographic isolation, as described for LCAT activity.

Triglyceride concentrations were determined according to the semi-automated method of Royer and Ko (1969), adapted for use with the AutoAnalyzer.

Lipoprotein Profile

At the beginning and at the end of the study, one sample of fresh plasma from each subject was sent to the Good Samaritan Hospital laboratory for the lipoprotein scan. The major lipoprotein classes were separated electrophoretically and are expressed as percent of total lipoproteins.

Phospholipids

Phosphatidyl choline and lysophosphatidyl choline determinations were made on plasma samples from 12 subjects. The choice of subjects was made by selecting 12 numbers from 1-31 from a table of random numbers (Dixon and Massey, 1969); the plasma of correspondingly code-numbered subjects was analyzed. The code numbers of the two subjects who had suffered heart attacks were eliminated from the selection process.

The measurement of lysolecithin and lecithin required extracting

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[6]Cholesterol and triglyceride analyses were performed by L. Blecher.

[7]Technicon Corp., Tarrytown, NY

total lipids, separating the phospholipids by thin layer chromatography and analyzing the PC and LPC spots for phosphorus content by spectrophotometry. The method described by Chiu (1969) was used for lipid extraction. The lipids in 1 ml of rapidly thawed plasma were extracted into chloroform/methanol (2:1); this solution was evaporated under N_2 to a volume of about 1 ml and transferred quantitatively to a 1 ml volumetric flask. The volume was brought to 1 ml with chloroform/methanol.

The two lipid extracts of each subjects' plasmas before supplementation were combined, as were the two plasma samples taken after supplementation. Fifty microliters of the combined extracts were spotted on silica gel plates (0.25 mm, 20 x 20 cm) which had been activated for one hour at 115°C. The plates were developed in a solution of chloroform/methanol/acetic acid/water, 25:15:4:2. Individual phospholipids were located on the plate by exposure to iodine vapors, and identified by comparison with the chromatograms of standard phospholipids. Lecithin and lysolecithin spots were suctioned into chloroform/methanol and filtered. The filtrate was evaporated under N_2 and the phospholipids were redissolved in 1 ml of chloroform. One hundred microliters of the PC and 300 microliters of the LPC were pipetted in triplicate into 7 x 70 acid-washed tubes. These extracts were evaporated to dryness; 50 ul of ashing solution (0.4 N $HClO_4$ in 5 N H_2SO_4) were added to the samples and to a set of standards containing 0.15, 0.10, 0.05, and 0.025 ug phosphorus. All the tubes were dried for two hours at 95°C and then ashed for two hours at 165°C. Phosphorus was reacted with a color reagent, consisting of one part 10% ascorbic acid to nine parts 0.25% ammonium molybdate in 0.1 N sodium acetate. Three hundred microliters of color reagent were added to each tube. The tubes were incubated for two hours at 37° to develop the color. The optical density, which was proportional to phosphorus content, was read at 820 nanometers on a Beckman Model DU spectrophotometer.

Statistical Treatment

A paired t-test (Dixon and Massey, 1969) was used to compare the pre- and post-treatment values for dietary components, lipid con-

centrations, LCAT activity, platelet aggregation, and recalcified plasma clotting time. In analyses in which two values were obtained before and two after, the paired-*t*-test was between averages of the pre- and post-treatment measurements; when data were missing for one of these four points, that subject's values were omitted from the *t*-test. Correlation coefficients between variables were computed for both periods (Snedecor and Cochran, 1973).

RESULTS

Subjects

The mean age of the 30 subjects was 49 years, with a range of 38 to 59 years (Table 1). The mean activity level, on a scale of one to four, was 1.9. Thus, the group in general tended to be more sedentary than active. Their weights averaged 5% above the desirable weight for height (Metropolitan Life Insurance Statistical Bulletin, 1959) and ranged from 87 to 127% of desirable weight (Table 1). No consideration of body build or fatness was made in estimating relative weights. Two of the subjects were cigarette smokers and two were pipe smokers.

Dietary Intake

Table 2 shows the mean kilocalories, total fat, cholesterol, and linoleic:saturated fatty acid ratio of the diets at the beginning and at the end of the experimental period. There were no statistically significant differences in these dietary components recorded before and after the study, except for cholesterol, which increased from 311 mg to 395 mg per day ($p < 0.025$). Both levels are remarkably low compared to the typical U.S. intake and close to the 300 mg/day recommended by the Select Committee on Nutrition and Human Needs of the U.S. Senate (1977).

Fat constituted 37.6% of the calories. This proportion is lower than the estimated 42% in the current U.S. diet but considerably higher than the 30% recommended by the Senate Select Committee. The calculated ratio of linoleic to saturated fatty acids in the subjects' diets was 0.39, as contrasted to 0.41, reported for the U.S. diet in 1972. Both ratios fall short of the 1:1 ratio of polyunsaturated to saturated fatty acids recommended by the Senate Select Committee.

Mean nutrient intakes (shown in Appendix i) were well above the recommended dietary allowances (Food and Nutrition Board, 1974). However, a number of individual two-day diet records showed less than two-thirds of the recommended allowances for specific nutrients.

Table 1. Description of subjects.

	Mean[a] ± S.D.	Range
Age - years	49 ± 6	38 - 59
Weight - kg	78.3 ± 9.3	62.6 - 93.4
Height - cm	179 ± 7	168 - 193
Relative weight[b] - %	105 ± 10	87 - 127
Activity level[c]	1.9 ± 0.7	1 - 3

[a]Average of 30 subjects participating in the study.

[b]Percent of ideal weight for height from tables of weight standards for men (Metropolitan Life Insurance Statistical Bulletin, 1959).

[c]1=sedentary, 2=light activity, 3=moderate activity, 4=strenuous activity (Bogert et al., 1973).

Table 2. Mean intake of selected dietary components before and after lecithin supplementation.

	Before[a]	After[b]	Average[c]
Kilocalories	2209	2245	2226
Fat - gm.	90	96	93
Cholesterol - mg	311	395[d]	351
Linoleic:saturated fatty acid	0.39	0.39	0.39

[a]Mean of 29 two-day dietary records.

[b]Mean of 26 two-day dietary records.

[c]Mean of 55 two-day dietary records.

[d]Significant increase at $p < .025$, n= 26.

Eight of the 55 diets were low in vitamin A, seven were low in calcium, and four were low in thiamin.

Lecithin Capsules

Analysis of the lecithin capsules yielded the following phospholipids, reported as percent of total phosphorus in the capsule: lysophosphatidyl choline 0.1%, phosphatidyl ethanolamine 39.6 %, phosphatidyl choline 47.3%, phosphatidyl serine + phosphatidyl inositol 12.9%.

Plasma Lipids

Mean plasma lipid concentrations were within normal ranges both before and after lecithin supplementation (Table 3). Mean values for total cholesterol and cholesterol ester are almost identical to those reported for middle-aged men by Holden (1976), who used the same laboratory methods and many of the same subjects. Plasma triglycerides were slightly lower than those found in the Holden study. The mean PC concentration in the current study was comparable to values reported for middle-aged males by Halvorsen (1976) and by Böttinger (1973). However, the LPC concentration of 3.6 ug P/ml was very much lower than the 10.6 and 11.8 ug P/ml observed by Halvorsen and Böttinger, respectively.

After six weeks of lecithin supplementation, no change was observed in mean plasma concentrations of cholesterol, cholesterol ester, or LPC. The decrease in plasma PC was not statistically significant. Triglycerides, on the other hand, had increased significantly ($p<0.005$) at the end of the study.

Table 4 shows the correlation coefficients among the plasma lipids before and after lecithin supplementation. Plasma PC concentrations were positively correlated with total cholesterol, free cholesterol, and cholesterol ester. No significant correlations were seen between LPC and other lipids.

Plasma lipoprotein fractions were evaluated according to the normal ranges in use at the hospital laboratory where the analyses were performed. These ranges are: HDL 3-25% of total lipoprotein, LDL 40-65%, VLDL 10-25%, and chylomicrons 0-5%. As shown in Table

Table 3. Plasma lipid levels, LCAT activity, and coagulability before and after lecithin supplementation.

Variable	n	Before	After	Percent Difference	Significance
Total cholesterol, mg/dl	29	202.5	194.7	-3.9	n.s.[a]
Cholesterol ester, %	29	67.3	67.0	-0.4	n.s.
Cholesterol ester, mg/dl	29	136	131	-3.7	n.s.
Free cholesterol, mg/dl	29	66.3	64.1	-3.3	n.s.
Triglycerides, mg/dl	29	75.9	90.2	18.8	$p < 0.005$
Phosphatidyl choline, ug P/ml	12	71.2	66.5	-6.6	n.s.
Lysophosphatidyl choline, ug P/ml	12	3.56	3.21	-9.8	n.s.
HDL, %	30	30.9	30.7	-0.6	n.s.
LDL, %	30	47.1	44.3	-5.9	$p < 0.025$
VLDL, %	30	21.3	24.0	12.7	n.s.
Fractional LCAT, %/hr	29	3.45	4.26	23.5	$p < 0.005$
Molar LCAT, umole/L/hr	29	59.9	71.8	19.9	$p < 0.005$
Platelet aggregation, %	29	73.6	75.9	3.1	n.s.
Recalcified plasma clotting time, seconds	29	105	98	-6.7	$p < 0.01$

[a] n.s. indicates no significant difference at $p < 0.05$.

Table 4. Correlations among plasma lipids before and after lecithin supplementation.

		HDL	LDL	VLDL	PC
Relative weight	before	-0.456[a]	0.156	0.147	0.339
	after	-0.415[a]	-0.022	0.331	0.217
Total cholesterol	before	-0.311	-0.049	0.334	0.666[a]
	after	-0.134	-0.072	0.230	0.671[a]
Free cholesterol	before	-0.244	0.025	0.207	0.638[a]
	after	-0.120	0.071	0.041	0.586[a]
Cholesterol ester	before	-0.333	0.384[a]	-0.084	0.652[a]
	after	-0.136	0.298	-0.123	0.694[b]
PC	before	-0.078	0.268	0.088	
	after	-0.389	0.771[b]	-0.260	
LPC	before	-0.072	0.195	-0.305	0.018
	after	0.177	0.464	-0.448	0.498
Triglycerides	before	-0.588[b]	-0.722[b]	0.548[b]	0.487
	after	-0.667[b]	-0.661[b]	0.700[b]	0.525
LDL	before	-0.175		-0.722[b]	
	after	-0.063		-0.661[b]	
VLDL	before	-0.515[b]			
	after	-0.679[b]			

[a]Significant at $p < 0.05$.

[b]Significant at $p < 0.01$.

3, our subjects had an unusually high proportion of HDL and a relatively low proportion of LDL; the VLDL fraction was in the high-normal range. Our data do not include the lipid content of the fractions.

The percentages of HDL were negatively correlated with relative weight and with TG, both before and after lecithin supplementation (Table 4). The VLDL fractions were negatively correlated with the HDL and LDL before and after supplementation, but positively correlated with the TG.

Since several studies have shown an effect of lecithin on blood lipids of hyperlipidemic subjects (Morrison, 1958; Svanberg et al., 1974), changes in plasma lipids in our subjects with the highest lipid levels were analyzed. According to a handbook for physicians and dietitians published by the National Heart and Lung Institute (DHEW, 1974), "Hyperlipidemia deserving some attention may exist when cholesterol exceeds 220 mg/dl or triglyceride exceeds 150 mg/dl." We examined the average pre- and post-treatment lipid concentrations of each subject. There were 12 subjects with cholesterol greater than 220 mg/dl, three with TG above 150 mg/dl but 11 with TG above 100 mg/dl. For 13 subjects, the LDL exceeded 50% of the lipoproteins. Analysis of these values follows:

	n	Before	After	Change	Significance
Mean TC of subjects with $>$ 220 mg/dl -mg/dl	12	230.2	221.7	-8.5	n.s.
Mean TG of subjects with $>$ 100 mg/dl -mg/dl	11	103.1	135.3	32.2	$p < 0.005$
Mean LDL of subjects with $>$ 50% -%	13	57.5	49.2	-3.7	n.s.

The subjects with high cholesterol corresponded to the group as a whole with a small, non-significant decrease in total cholesterol in response to lecithin supplementation. The subjects with triglycerides greater than 100 mg/dl had an increase of 32 mg/dl, which

was 31% of their initial TG level. This change was greater than the increase of 14.3 mg/dl, or 19% of the initial TG level, which was seen in the overall group. The decrease of 3.7% in the LDL was not significant at the 5% level. The group as a whole had a small but significant decrease in LDL.

LCAT Activity

The LCAT activity is reported two ways (Table 3). The fractional LCAT rate gives the percent of the ^{14}C-cholesterol present in the plasma which was converted to ^{14}C-cholesterol ester in one hour. The molar LCAT rate is the product of the free cholesterol concentration and the fractional LCAT rate; it is expressed as micromoles/L/hr. The fractional LCAT rates observed in this study were lower than the values of 6.4 $\pm$ 1.3%/hr that Wallentin et al. (1978) reported for normal subjects. After lecithin supplementation, there was a highly significant increase ($p < 0.005$) in both the fractional and molar LCAT rates. Fractional LCAT rate was negatively correlated with total cholesterol, free cholesterol, and cholesterol ester (Table 5). Molar LCAT was positively correlated with TC, FC, CE, and PC before and after lecithin supplementation, with TG after, and with relative weight before the experimental period.

Coagulability

Recalcified plasma clotting times decreased from 105 seconds to 98 seconds ($p < 0.01$). Normal clotting times for this method using platelet rich plasma are 90 to 120 seconds (Davidsohn and Wells, 1962). Clotting times were not significantly correlated with plasma phospholipid concentrations.

Platelet aggregation was recorded as the percent of aggregation of platelet-rich plasma induced by 1.2×10^{-5} M ADP. Platelet aggregation increased from 73.6% to 75.9%, but this was not a significant change at the 5% level of significance (Table 3). Platelet aggregation was not significantly correlated with plasma lipid levels.

Table 5. Correlations between LCAT activity, plasma lipids, and relative weight.

		Fractional LCAT	Molar LCAT
Total cholesterol	before	-0.452[b]	0.434[a]
	after	-0.433[a]	0.494[b]
Free cholesterol	before	-0.506[b]	0.405[a]
	after	-0.498[b]	0.464[b]
Cholesterol ester	before	-0.407[a]	0.431[a]
	after	-0.390[a]	0.494[b]
Phosphatidyl choline	before	0.114	0.626[a]
	after	0.107	0.591[a]
Lysophosphatidyl choline	before	0.092	0.254
	after	0.327	0.524
Triglycerides	before	0.144	0.328
	after	0.244	0.589[b]
Relative weight	before	0.352	0.403[a]
	after	0.059	0.286

[a]Significant at $p < 0.05$.

[b]Significant at $p < 0.01$.

DISCUSSION

Subjects

Middle-aged males were the chosen population group for this study because they exhibit a high prevalence of cardiovascular disease. Our subjects were volunteers, many of whom expressed an interest in the aims of this research as well as a concern for their own health. As a result of this health-consciousness, the group was biased toward health maintenance practices, such as weight control, regular physical activity, abstention from smoking, and selective dietary intake. This probably accounted for the near-ideal weights, low levels of dietary calories, fat, and cholesterol, and low plasma lipid concentrations.

Diets

The subjects were asked to maintain their normal diets throughout the study so that any changes which were observed could be clearly ascribed to the effect of lecithin supplementation and not to dietary factors. Nevertheless, a certain amount of day-to-day variability in dietary intake was expected and was seen in calories, fat, linoleic/saturated fatty acid ratio, and other nutrients. The small increase in cholesterol intake was statistically significant ($p < 0.025$). However, dietary cholesterol was not correlated significantly with any of the plasma factors studied and thus the small increase noted can probably be ignored.

Phospholipids in Diet and Plasma

Despite daily oral supplementation of 10.8 g lecithin, 47.3% of which was phosphatidyl choline, the mean plasma PC decreased, although not significantly. One may estimate that the usual daily intake of lecithin is 3 to 6 g (Bogert et al., 1973) thus 10.8 g of supplemental lecithin amounts to a considerable addition.

Lecithin is well-absorbed after conversion to lysolecithin in the small intestine (Nilsson and Borgstrom, 1967; Rodgers et al.,

1975), and it is expected that additional lecithin provided by the capsules was almost completely absorbed. However, an increased amount of lecithin of dietary or biliary origin may decrease the de novo synthesis of PC from choline by the intestinal mucosa. This adaptation maintains the molar ratio of PC to TG in chylomicrons (Mansbach, 1977). A reduced de novo synthesis of lecithin may explain why dietary lecithin supplements did not alter plasma PC concentrations. Halvorsen (1976) found no significant change in plasma phospholipids after daily supplementation with 7.2 g lecithin, nor did Enticknap (1962) upon supplementation with 0.6 to 1.8 g lecithin. Wallentin (1977a) reported an increase in plasma phospholipid when lecithin was injected intravenously but not when it was given orally. Oral lecithin feeding led to a dose-related increase in serum free choline (Wurtman et al., 1977). This free choline in the blood could be available to tissues for synthesis of PC or acetylcholine. Perhaps circulating free choline serves to maintain a balance between the diet-supplied PC, the endogenous production of PC, and the tissue requirements for PC.

The LPC levels in the current study were much lower than those reported by Böttinger (1973), Robinson and Philips (1962), and Halvorsen (1976), all of whom used similar thin layer chromatography methods. The former two studies used serum; the time allowed for coagulation to occur (up to one hour) in serum samples also permits LCAT activity to proceed at rates of approximately 60-90 umole/L/hr. This activity can produce an additional 1.9 to 2.8 ug P/ml/hr in the LPC fraction. Halvorsen analyzed plasma rather than serum, but the time taken for thawing may have been lengthy. A rapid thawing of samples before lipid extraction prior to phospholipid analysis is equally as important as rapid freezing in minimizing the effect of LCAT on LPC levels. The LCAT action simultaneously diminishes PC levels, though the effect is relatively less drastic, since the amount of PC is much greater than LPC.

Dobiasova et al. (1976) theorized that LPC has an atherogenic effect by lowering the affinity of cholesterol for lipoproteins and

increasing cholesterol influx and accumulation in body organs. Portman and Alexander (1969) reported that atherosclerotic monkeys had much higher plasma and aortic concentrations of LPC than normal monkeys. Elevated LPC may have been due to increased LCAT activity. They found that LPC was rapidly removed from the plasma by the aorta, where it was acylated to PC. If elevated plasma LPC is an indicator of atherosclerosis in humans, the finding of low LPC in our subjects would be a favorable indication. On the other hand, plasma LPC was reported to be lower in patients with ischemic heart disease than in normals (Gillett and Besterman, 1975).

Plasma Lipids

As in Holden's study (1976) we found no change in plasma total cholesterol following lecithin supplementation. No significant decrease in TC was seen in the subjects who had greater than 220 mg/dl cholesterol. Thus our results do no support those of Morrison (1958) that lecithin lowers TC in hypercholesterolemic subjects. Our findings of no change in percent cholesterol ester do not repeat those of Holden that percent CE increased significantly by 2% after lecithin supplementation. In light of the findings of Schwartz et al. (1978) that the liver preferentially extracts FC rather than CE from HDL for bile cholesterol synthesis, the assumption that CE is the form in which cholesterol is transported for removal from plasma loses strength. If free cholesterol is more readily removed than CE by the liver, then higher CE levels may not be favorable for prevention of cholesterol accumulation in body tissues. However, Rosseneu et al. (1979) showed an increase in CE and LPC in HDL_3 and VLDL after adding polyunsaturated PC to the diet of chimpanzees. They postulated that such lipoprotein alterations made the surface more fluid and the core denser and resulted in more efficient transport of cholesterol away from cells.

Svanberg et al. (1974) found a 16% decrease in triglycerides due to lecithin supplementation to hypertriglyceridemic subjects, but the normolipidemic subjects of the current study showed an opposite effect. The 19% increase in mean triglycerides ($p < 0.005$) of our subjects

was similar to the 16% increase reported by Holden (1976), although the latter was not statistically significant. Subjects with the highest levels of triglycerides showed an even greater increase, 31% of their initial TG. However, the group's mean TG was only 75.9 mg/dl initially and 90.2 mg/dl after the experimental period; both means fell within the normal range of 30-135 mg/dl (Henry et al., 1974). Since the dietary fat did not increase, the source of the increased plasma TG must have been either increased endogenous secretion, or decreased uptake by the cells. The subjects' triglycerides underwent large day-to day fluctuations. When comparisons were made of TG levels in plasma samples taken three days apart, there was a mean difference of 16.6 mg/dl, with a t value of 3.16 ($p < 0.005$). The increase in TG seen after the experimental period was possibly partially accounted for by normal fluctuations.

Lipoproteins

HDL contains about 30% phospholipid, the greatest proportion of any of the lipoproteins. An increased supply of lecithin to the plasma by dietary supplementation might by reckoned to increase the amount of HDL in the blood. However, we did not see any change in HDL, and the HDL levels were not significantly correlated with plasma PC levels in the subjects for whom PC values were available. Childs et al. (1977) reported an increase in HDL cholesterol in females, but no change in normolipidemic males with lecithin supplementation. This would seem to concur with our data showing no change in the proportions of lipoproteins as HDL.

The principal lipid in LDL is cholesterol. Although the proportion of LDL declined significantly after lecithin supplementation, total cholesterol did not decrease significantly (Table 3) and the LDL proportions were not significantly correlated with TC (Table 4). Childs et al. (1977) reported a 7% decrease in LDL cholesterol but no change in TC after lecithin supplementation. These results imply that cholesterol is redistributed among the lipoproteins. The cholesterol concentrations of lipoprotein fractions were not determined in our study, but no change was observed in the proportion of

lipoproteins as HDL and only a slight and statistically non-significant increase in VLDL. The only evident trend in lipoprotein reproportioning due to lecithin in the subjects was the decreased LDL.

The VLDL levels were negatively correlated with both HDL and LDL (Table 4). As expected, the percent VLDL was positively correlated with its main constituent, TG. TG was negatively correlated with HDL, in which it is a minor component. Evidently, subjects with high levels of HDL tended to have low VLDL and TG. This is consistent with the concept that high HDL cholesterol is protective against cardiovascular disease, while elevated TG is a risk factor for the disease.

LCAT

The finding by Holden (1976) of an 11% increase in LCAT activity following 7.2 g lecithin supplementation was confirmed by the current research. With a lecithin dose of 10.8 g, the 30 subjects showed a 23% increase in fractional LCAT and a 20% increase in molar LCAT rates (Table 3). Lecithin appears to have a dose-related effect on LCAT activity.

The fractional LCAT, a measure of the percent of the free cholesterol which is esterified per hour, was negatively correlated with the concentration of its substrate FC and not significantly with plasma PC, the second reactant (Table 5). Thus, subjects with higher plasma free cholesterol did not have a correspondingly greater capacity to esterify it. In fact, the esterification rate was proportionately lower than for those with low plasma FC. This situation would be expected to produce a lower percent CE in those with higher FC and lower fractional LCAT, but the percent of CE was not significantly correlated with either fractional LCAT or FC. The fractional LCAT was negatively correlated with the absolute amount of CE, suggesting feedback inhibition of the enzyme. However, LPC, which is an inhibitor of LCAT activity, was not significantly correlated with either the fractional or molar rates. The percent CE remained at 67 $\pm$ 2 % before and after the experimental period (Table 3) in spite of the increase in LCAT, which suggests a control mechanism to maintain the

percent CE at a constant level. Rose and Juliano (1979) showed that CE was transferred from HDL_3 and very high density lipoproteins to VLDL in vitro. This non-enzymatic transfer ranged from 2.9 to 13.8 mg/dl of HDL-CE over 12 hours, which is 65 to 310 umole/L/hr. This rate is faster than the mean esterification rates of about 60 to 72 umole/L/hr in the fasting plasmas of our subjects. Such a transfer could serve as the major exit for the LCAT-produced CE on the HDL. Since the proportion of cholesterol ester remained constant in spite of the greater LCAT activity, the CE produced by the LCAT reaction must be removed from the lipoproteins by the tissues or the liver.

The molar LCAT, which is the product of the free cholesterol concentration and the fractional LCAT rate, was positively correlated with its substrates FC and PC (Table 5). CE also was positively correlated with molar LCAT, but this was mainly due to the high correlation of molar LCAT with plasma free cholesterol concentration and to the constant percent of cholesterol as CE. The positive correlation between molar LCAT rates and FC and phospholipids is in accord with the findings of Wallentin et al. (1978). These investigators also reported a correlation between molar LCAT and TG and VLDL. The positive correlation between molar LCAT and relative weight, reported by Wallentin (1977b) and by Dobiasova and Vondra (1978) was also seen in our subjects prior to the experimental period.

The effect of increased LCAT is to form more CE and LPC, using PC and FC. The CE may be transferred to the VLDL in exchange for TG (Rose and Juliano, 1979). The increased CE on the VLDL surface reduces surface stability and allows penetration by lipoprotein lipase to the TG core; the CE thus may aid in lipolysis of VLDL triglycerides (Cramp and Tickner, 1978). The LPC produced by LCAT may be picked up by an albumin-apo A-I lipoprotein fraction and transported to various tissues for reconversion to PC (Wille et al., 1978).

LCAT has a necessary role in lipoprotein metabolism. The absence of the enzyme due to an inborn gene error produces severe consequences, including elevated FC, PC, and TG, and premature atherosclerosis (Norum, 1973). Yet, activation of the enzyme by lecithin supple-

mentation does not necessarily help to prevent atherosclerosis. Wallentin et al. (1978) found that in type II and type IV hyperlipoproteinemias, molar LCAT rates were elevated, but fractional LCAT rates were lower than normal for type II and were higher than normal for type IV patients. It is not clear why LCAT is high in type IV where triglycerides are elevated, but when LCAT is absent due to a genetic disorder, triglycerides are also very high. One may conclude that proper functioning of the enzyme is important in maintaining a balance of lipids in the blood.

Platelet Aggregation

The aggregability of platelets has been shown to be increased in the plasma of subjects with cardiovascular disease. Bolton et al. (1967) showed that elevated LDL-LPC in arterial desease conferred abnormal sensitivity of platelets to ADP-induced aggregation. Likewise, Olcott and Wylie (1977) reported an increased platelet sensitivity to ADP in severe atherosclerosis, but not in mild forms of the disease. Platelets from type II hyperlipoproteinemic subjects were also much more sensitive to ADP-induced aggregation (Carvalho et al., 1974). The measurement of sensitivity of platelets to aggregating agents is commonly made by serial additions of ADP to the plasma until reversible, biphasic, or irreversible aggregation is detected. Platelet sensitivity to a series of ADP additions was not a practical form of measurement in a study with as many as 30 subjects since such a process is very time-consuming and the fresh plasma must be tested before three hours have expired. Consequently, our results are reported as percent aggregation induced by a high ADP concentration to which the response was usually irreversible aggregation, although a biphasic response was seen in some plasma samples. Because the parameter measured was not identical to the afore-mentioned studies, a close comparison of results is not valid.

While Besterman and Gillett (1971, 1973) reported a dose-dependent decrease in secondary platelet aggregation due to LPC, no correlation was seen between platelet aggregation and either LPC or PC in the plasma of our subjects. In contrast to the decreased sensitivity

of platelets to ADP-induced aggregation when lecithin was added to the diet of hyperlipidemic guinea pigs (Cusack, 1978), the human subjects in this study showed no change in ADP-induced aggregation. Miller and Nordøy (1977) found no effect on platelet aggregation when additions of VLDL, LDL, and HDL were made to plasma in vitro. This is confirmed by the current study; there was no significant correlation of platelet aggregation with any of the plasma lipids or lipoproteins measured. Thus, lecithin supplementation in normolipidemic human subjects appears to have no effect on the extent of platelet aggregation induced by ADP.

Recalcified Plasma Clotting Time

A decrease in the plasma clotting time followed lecithin supplementation and this indicates a tendency toward either more rapid thrombus formation or slower lysis of the clot as it forms. If the result of additional lecithin is hypercoagulability, as is suggested by the shortened clotting time, oral lecithin should be avoided rather than encouraged for prevention of cardiovascular disease.

A decrease in clotting time during postprandial hyperlipemia is a common clinical observation. This is consistent with our finding that clotting time decreased while triglyceride concentration increased with treatment. However, the clotting times were positively correlated with the fasting concentrations of TG before treatment, and with VLDL before and after the experimental period.

It can be speculated that the effect on clotting by lecithin supplementation was due to changes in platelet phospholipids. Marcus (1966) reported that the addition of phospholipids to platelets in vitro promoted clot formation by producing a negative surface charge on the platelets. Phospholipids are also a requirement in the formation of an intrinsic activator of prothrombin in the series of reactions which occur in the clotting mechanism. The mixture of phospholipids in the lecithin capsules could have increased the availability of phospholipids in the plasma and activated the clotting mechanism. The clotting time was not significantly correlated with

plasma PC or LPC, but correlations with other plasma phospholipids remain as untested possibilities.

Concluding Remarks

Both the mean LCAT rates and the triglyceride concentrations increased after lecithin treatment. Possible reasons for the higher enzyme activity include greater secretion of LCAT by the liver, greater activation by substrates or apolipoproteins, or decreased inhibition by apolipoproteins. It is possible that more LCAT was synthesized in response to the rise in plasma TG. Nordby and Norum (1978) noted that LCAT synthesis by rat hepatocytes was high when the secretion of TG was also high. Despite the change observed in LCAT rate, no change was seen in the concentrations of either substrates or products. This suggests that the turnover of the substrates, free cholesterol and phosphatidyl choline, was higher, and that the plasma concentrations of the products, cholesterol ester and lysophosphatidyl choline, were maintained by greater extraction from plasma by the tissues. If, as a result of increased cholesterol turnover, cholesterol was drawn from the tissues and transported as CE to the liver for bile production, this mechanism would be a means of disposing of tissue cholesterol. But if the CE produced is picked up by tissues other than the liver, the effect could be an increase in the pool of tissue cholesterol. Further research should be done using radioactively labelled cholesterol in animals to determine which tissues extract the LCAT-produced CE.

The responses to lecithin supplementation of the two measures of coagulability were not consistent with each other. While clotting time was shortened, platelet aggregation did not change significantly. Since the mean clotting times both before and after treatment fell within the normal range, the observed effect may be of concern only to those with a tendency toward hypercoagulability.

SUMMARY

Daily addition of 10.8 g of lecithin to the diets of 30 normolipidemic middle-aged males for six weeks was shown to have a number of effects on factors related to lipid metabolism and atherosclerosis. The lipid and lipoprotein levels of the group studied suggested that these subjects were at relatively low risk for development of cardiovascular disease. Lecithin produced two significant alterations in their lipid profiles: an increase in triglycerides and a decrease in LDL. Such a response is equivocal in its relation to atherosclerosis; the former effect increases risk of the disease, while the latter reduces the risk. No changes in phosphatidyl choline, lysophosphatidyl choline, total cholesterol, free cholesterol, or cholesterol ester were noted. Since plasma TG and LCAT activity both increased with the treatment, but no relation was identified between LCAT and any of the lipoproteins, there is no indication that the increase in LCAT activity is advantageous.

Lecithin supplementation produced no change in the response of of platelets to ADP-induced aggregation. Aggregability was not correlated with plasma lipids or phospholipids. On the other hand, the mean recalcified plasma clotting time decreased after supplementation. This increased coagulability may have been due to the effects of the supplemental phospholipid mixture on the clotting process. Increased coagulability would not be a desirable goal in the treatment of cardiovascular disease.

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APPENDIX

Appendix i. Mean nutrient intakes calculated from two (two-day) diet records before and after lecithin supplementation.

Subject Number	Kcal	Protein	Fat	Carbo-hydrate	Ca	Fe	Vit. A	Thiamin	Ribo-flavin	Niacin	Vit. C	Linoleic/ Sat. F.A.	Cholesterol
		g	g	g	mg	mg	IU	mg	mg	mg	mg		mg
1	2776	92	128	313	1158	19.6	24114	1.94	3.06	30.3	83	.39	624
2	2262	127	97	154	631	19.2	2695	0.79	1.45	25.6	95	.10	456
3	2238	90	102	261	1111	13.2	3205	1.55	1.83	18.4	73	.28	261
4	2248	100	84	229	514	18.5	4401	1.14	1.53	28.9	150	.48	215
5	2155	77	76	318	905	17.3	8343	1.40	1.69	18.5	215	.35	318
6	2661	95	129	241	923	13.0	4295	1.48	1.93	26.6	164	.46	337
7	2382	95	100	300	1375	12.8	5395	1.46	2.26	19.6	60	.24	232
8	2883	112	122	347	1458	21.0	26928	2.02	3.85	27.4	358	.53	376
9	2226	84	89	268	1060	14.2	3303	0.95	1.88	14.5	161	.29	306
10	1773	84	77	193	642	13.7	3034	0.97	1.27	17.7	83	.33	264
11	2072	71	99	205	769	11.9	5926	1.07	1.70	14.1	145	.33	352
12	1514	64	55	197	613	9.9	2904	0.76	1.09	16.8	149	.32	161
13[a]	1806	68	73	198	853	9.5	3232	0.73	1.41	14.9	30	.26	187
14[a]	2328	73	103	311	860	14.5	13314	1.40	1.59	12.9	101	.24	536
16	2857	96	123	351	925	17.1	7783	1.53	1.86	19.7	106	.33	298
17	3643	139	168	365	1955	20.8	11171	1.96	3.24	27.3	221	.71	557
18	2224	73	105	251	610	14.3	6950	1.46	1.39	15.8	158	.57	209
19	1336	76	57	142	747	12.8	9132	1.05	1.44	16.4	218	.67	252
21	1979	109	77	206	841	18.4	31687	1.23	3.37	23.8	224	.24	524
22	2093	79	102	201	537	9.7	6152	1.43	1.28	20.0	363	.45	314
23	2336	107	103	229	1598	15.0	22008	1.34	3.90	21.3	118	.36	386
24	2680	110	81	390	856	20.9	7287	1.55	1.89	25.8	125	.32	467
25	1508	90	52	167	1001	11.0	4732	1.06	1.89	21.1	88	.59	349
26	2083	112	84	171	942	16.2	5554	1.90	2.03	24.8	115	.19	386
27	2342	76	76	351	664	10.4	8629	1.06	1.54	21.9	150	.73	181

Appendix i. (continued)

Subject Number	Kcal	Protein	Fat	Carbo-hydrate	Ca	Fe	Vit. A	Thiamin	Ribo-flavin	Niacin	Vit. C	Linoleic/ Sat. F.A.	Cholesterol
		g	g	g	mg	mg	IU	mg	mg	mg	mg		mg
28	2122	96	78	272	948	31.3	13078	1.23	1.86	17.8	140	.36	399
29	1678	70	57	220	822	13.0	4431	1.00	1.58	9.0	160	.27	490
30	2145	104	97	209	885	15.0	7884	1.96	1.83	20.0	141	.30	481
31[a]	1824	89	83	170	493	9.9	4122	0.92	1.14	17.2	55	.40	244
Mean	2225	93	93	250	930	15.5	9183	1.34	1.99	20.2	151	.35	350

[a]Intakes for only one three-day diet record.

Appendix ii. Plasma LCAT activity as per cent cholesterol esterified per hour in subjects before and after lecithin supplementation.

Subject Number	Before			After		
	A	B	Average	A	B	Average
1	3.95	3.39	3.67	4.80	4.43	4.62
2	2.35	1.81	2.08	4.15	3.94	4.05
3	7.29	4.61	5.94	5.26	8.14	6.78
4	4.46	3.01	3.74	3.98	5.44	4.71
5	3.91	1.66	2.79	2.92	3.94	3.43
6	4.73	4.40	4.57	3.65	4.56	4.11
7	3.14	2.78	2.96	6.41	5.19	5.80
8	2.47	3.07	2.77	2.65	3.56	3.11
9	4.38	1.41	2.90	4.42	4.88	4.65
10	2.43	4.83	3.63	5.73	5.41	5.57
11	4.80	4.04	4.42	5.81	4.73	5.27
12	3.67	3.27	3.47	3.68	4.06	3.87
13	4.23	2.44	3.34	3.72	5.14	4.43
14	3.01	4.07	3.54	2.77	4.20	3.49
15	3.06	1.65	2.36	3.44	3.20	3.32
16	4.19	4.82	4.51	5.57	. . .	5.57
17	3.12	2.44	2.78	2.66	3.96	3.31
18	3.50	1.55	2.53	4.93	4.01	4.47
19	3.68	3.44	3.56	4.13	3.67	3.90
21	3.22	4.45	3.84	4.20	4.60	4.40
22	3.22	3.75	3.49	3.02	2.95	2.99
23	3.97	3.68	3.83	2.78	4.46	3.62
24	2.13	2.79	2.46	2.91	5.10	4.01
25	5.74	2.20	3.97	3.88	4.29	4.09
26	3.76	1.57	2.67	3.92	3.52	3.72
27	3.63	1.16	2.40	4.21	2.91	3.56
28	4.99	1.77	3.38	3.13	4.03	3.58
29	3.40	4.38	3.89	6.58	5.63	6.11
30	4.18	4.44	4.31	2.80	4.29	3.55
31	3.85	5.71	4.78	3.80	6.56	5.18
Mean	3.82	3.15	3.49	4.06	4.50	4.31
S.D.	1.05	1.25	0.86	1.13	1.08	0.96

Appendix iii. Percent platelet aggregation induced by 1.2×10^{-5} M. ADP in subjects before and after lecithin supplementation.

Subject Number	Before			After		
	A	B	Average	C	D	Average
1	67.6	78.2	72.9	74.6	73.2	73.9
2	76.7	84.6	80.7	69.9	75.6	72.8
3	76.2	78.1	77.2	78.0	71.8	74.9
4	65.8	72.4	69.1	78.2	74.8	76.5
5	79.9	71.5	75.7	76.9	80.4	78.7
6	68.8	77.0	72.9	75.7	78.5	77.1
7	70.8	63.2	67.0	72.3	70.7	71.5
8	73.4	86.1	79.8	69.3	70.4	70.1
9	71.1	74.5	72.8	72.0	66.4	69.2
10	79.2	73.6	76.4	85.7	70.0	77.9
11	81.7	79.9	80.8	78.5	78.2	78.4
12	70.6	77.8	74.2	76.4	87.2	81.8
13	79.4	79.0	79.2	84.8	74.0	79.4
14	63.2	68.2	65.7	91.5	77.8	84.7
15	79.8	72.7	76.3	82.0	68.4	75.2
16	77.8	75.5	76.7	100.2	. . .	100.2
17	61.3	57.7	59.5	87.1	73.3	80.2
18	76.9	77.2	77.1	87.6	75.0	81.3
19	64.7	77.3	71.0	75.2	69.9	72.6
21	62.2	73.8	68.0	75.6	84.3	80.0
22	82.6	74.6	78.6	90.3	78.4	84.4
23	68.2	71.4	69.8	81.4	85.8	83.6
24	72.5	75.3	73.9	62.7	71.5	67.1
25	77.1	76.5	76.8	61.2	74.0	67.6
26	80.8	64.2	72.5	78.0	68.2	73.1
27	65.7	66.9	66.3	70.5	69.5	70.0
28	71.5	74.1	72.8	77.8	76.4	77.1
29	78.5	80.3	79.4	66.7	79.8	73.3
30	76.6	77.9	77.3	82.2	71.7	77.0
31	74.9	66.1	70.5	70.9	76.5	73.7
Mean	73.2	74.2	73.7	77.8	75.1	76.8
S.D.	6.3	6.2	5.1	8.6	5.3	6.5

Appendix iv. Recalcified plasma clotting times (in seconds) before and after lecithin supplementation.

Subject Number	Before			After		
	A	B	Average	C	D	Average
1	105	86	96	101	98	100
2	115	124	120	105	106	106
3	83	116	100	89	105	97
4	86	75	81	101	71	86
5	94	105	100	78	113	96
6	124	75	100	124	104	114
7	83	124	104	68	109	89
8	113	94	104	101	79	90
9	94	86	90	79	116	98
10	83	135	109	79	90	85
11	83	79	81	94	75	85
12	116	98	107	75	105	90
13	113	74	94	105	86	96
14	94	131	113	95	116	106
15	102	113	108	100	128	114
16	83	124	104	90	. . .	90
17	75	115	95	78	113	96
18	124	128	126	90	83	87
19	90	110	100	101	94	98
21	101	145	123	124	90	107
22	109	86	98	101	94	98
23	101	105	103	86	105	96
24	122	135	129	86	98	92
25	120	128	124	102	104	103
26	113	98	106	109	83	96
27	116	109	113	89	135	112
28	109	86	98	86	100	93
29	83	116	100	105	100	103
30	83	109	96	86	101	94
31	114	135	126	140	130	135
Mean	101	108	105	96	101	98
S.D.	15	21	12	16	16	11