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Title: EFFECT OF DIETARY LECITHIN ON CHOLESTEROL ESTERIFICATION, TISSUE CHOLESTEROL AND PLATELET AGGREGATION IN GUINEA PIGS

Abstract approved by: Elisabeth S. Yearick

Adult male guinea pigs were maintained on four dietary regimens for a period of 8 weeks. The basic diet consisted of powdered guinea pig chow with 10% coconut oil. The three experimental groups received the basic diet with additions of either 1% lecithin, 0.25% cholesterol, or 0.25% cholesterol plus 1% lecithin. Blood was sampled periodically for the determination of plasma cholesterol, phospholipids, lecithin:cholesterol acyltransferase activity (LCAT), and platelet aggregation. Animals were sacrificed at eight weeks and the concentrations of cholesterol in selected tissues and in the carcass were determined.

The cholesterol-fed animals gained less weight than did the controls; this was associated with reduced food consumption and also decreased feed efficiency.

The plasma lipids responded to cholesterol feeding with a sharp increase in total cholesterol, free cholesterol, total phospholipids, phosphatidyl choline (PC), and lysophosphatidyl choline (LPC).

The ratio of cholesterol:phospholipid increased, whereas the ratios of cholesterol ester:total cholesterol, LPC:total phospholipid, and LPC:PC declined. The reduced percentage of cholesterol ester and LPC suggested a limited ability to esterify the high levels of circulating cholesterol that accompanied cholesterol feeding. Although the net esterification (micromoles of cholesterol esterified) by LCAT increased with cholesterol treatment, the percent of plasma cholesterol esterified (fractional esterification) declined. Addition of lecithin to the cholesterol diet did not prevent the sharp rise in plasma cholesterol, phospholipids, or their fractions. The net esterification increased in plasma of animals receiving lecithin plus cholesterol. Nevertheless, the increase in LCAT activity was insufficient to maintain the proportion of esterified cholesterol at the level found in control animals. There was an increase in total phospholipid, PC, and LPC in plasma of guinea pigs supplemented with lecithin only; no change in cholesterol or cholesterol ester of plasma was seen. The molar esterification rate was positively correlated with plasma concentrations of both the substrates (free cholesterol and PC) and the products (cholesterol ester and LPC) of LCAT action. There was a strong positive correlation between the fractional esterification rate and the percent of esterified cholesterol, percent of LPC, and the ratio of LPC:PC.

Cholesterol treatment produced an increase in the relative sizes of spleen and liver, and definitely increased the cholesterol deposition in the total body as well as selected tissues (spleen, liver, kidney, lungs, digestive tract). The increased deposition in

the liver as well as total body was even greater when licithin was included in the atherogenic diet. Sudan staining of thoracic aorta revealed plaques especially around the aortic arch of animals receiving cholesterol only; the addition of lecithin to the atherogenic diet resulted in a reduced number of plaques.

ADP-induced platelet aggregation was enhanced with cholesterol feeding. The sensitivity of platelets to ADP aggregation was reduced in animals treated with lecithin plus cholesterol. The correspondence of platelet aggregation to the prevalence of aortic plaques suggests that contact with damaged vascular walls may have altered platelet sensitivity. Positive correlations existed between platelet aggregation and plasma concentrations of free and esterified cholesterol as well as PC and LPC.

The overall results suggest that the increase in the rate of cholesterol esterification and the decrease in the platelet sensitivity may be the mechanism by which lecithin reduces the incidence and severity of atherosclerosis.

EFFECT OF DIETARY LECITHIN ON CHOLESTEROL ESTERIFICATION, TISSUE CHOLESTEROL AND PLATELET AGGREGATION IN GUINEA PIGS

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INTRODUCTION

Lecithin has long been given attention as an agent to lower blood cholesterol and the incidence of atherosclerosis. Although many human as well as animal studies have been carried out thus far, the mechanism of action of lecithin with respect to cholesterol metabolism and atherogenesis remains unclarified.

Takeuchi and Yamamura (1973) observed increased LCAT (lecithin: cholesterol acyltransferase, E.C.2.3.1.26) activity in plasma of rats treated with 1% lecithin. It has been proposed that the increased percentage of linoleic acid in plasma esterified cholesterol as a result of polyunsaturated lecithin treatment may be responsible for the increased transesterification rate, enhancing the disposal of plasma cholesterol (Skorépa et al., 1974). A preliminary study in our laboratory with healthy middle-aged men who were receiving lecithin supplements revealed that an increase in LCAT activity tended to be accompanied by an increased ratio of lysophosphatidyl choline (LPC) to phosphatidyl choline (PC). LPC, the product of LCAT enzyme activity, has an exceptionally fast turnover rate in the plasma, due to its high affinity for plasma lipoproteins and cellular membranes (Portman and Alexander, 1976). If LPC rises as a result of increased LCAT activity with lecithin supplementation, it should, in turn, influence platelet behavior. Published reports generally relate enhanced platelet aggregability to the thrombotic complications of atherosclerosis.

Besterman and Gillett (1971) demonstrated an inhibitory action of LPC on in vitro platelet aggregation induced by ADP, epinephrine, and collagen, and postulated that LPC may be anti-thrombogenic.

This study was conducted to observe the time course of the effects of lecithin supplementation and cholesterol feeding on plasma cholesterol esterification and platelet aggregation in guinea pigs. Changes in the products as well as the substrates of LCAT action were monitored. ADP-induced platelet aggregation was also investigated in relation to the changes in plasma lipid components. In addition, an attempt was made to learn how changes in plasma lipid components with lecithin treatment are reflected in tissue cholesterol deposition.

Guinea pigs were chosen as the experimental animal because they resemble the human with respect to enzymes associated with cholesterol metabolism and platelet aggregation. Abnormalities in the cholesterol-fed guinea pigs strikingly resemble those seen in human familial LCAT deficiency (Stokke, 1974). In both conditions, there is deposition of free cholesterol in most of the tissues, culminating in atherosclerosis.

II. REVIEW OF LITERATURE

Studies on lecithin supplementation

The term "lecithin" is interchangeably used for two different materials. It is used to refer to a mixture of phospholipids which are soluble in most organic solvents but not in acetone. It is also used to refer to a specific phospholipid, phosphatidyl choline. Throughout this paper, "lecithin" will be used to describe the mixture of phosphorus-containing lipids and phosphatidyl choline will be described as PC.

Lecithin supplementation has long been thought to lower blood cholesterol and the incidence of atherosclerosis in both humans (Morrison, 1958; Andresen, 1965; Funatzu, 1966; Blaton et al., 1972; Skorépa et al., 1974; Svanberg et al., 1974) and various animal species (Friedman et al., 1957; Konecki et al., 1962; Abdulla et al., 1967; Kroupa et al., 1972; Patelski et al., 1970; Howard et al., 1971; Howard and Patelski, 1974; Waligora et al., 1975; Samochowiec et al., 1976a & b). Yet in many of the studies reported, the results are rather inconsistent (Davies and Murdoch, 1959; Butler et al., 1960; Enticknap, 1962; ter Welle et al., 1974). This may be due to variation in the following: the amount of lecithin administered, the fatty acid composition of lecithin, the length of the treatment period, the mode of administration, the choice of the experimental models, and the physiological state of the subjects.

Human studies

One of the early studies in humans was reported by Morrison (1958). The effect of oral lecithin supplementation was studied in twenty-one hypercholesteremic patients. The subjects were given 36 gm soybean lecithin daily with a low fat diet (<25 gm/day) for three months. At the end of the treatment period, plasma cholesterol decreased markedly and plasma phospholipids increased relatively, resulting in a significant decrease in the cholesterol to phospholipid ratio.

The therapeutic effect of polyunsaturated PC (trade names: Lipostabil, EPL) was investigated by Andresen (1965). Patients with hyperlipaemias of different origin were treated with 1,125 mg Lipostabil for two periods of two months each, with a placebo period between. A combination of intravenous and oral doses was given daily. During the total period of observation lasting six months, significant reduction of all lipid values was observed. The total cholesterol decreased by 29.2% in the first treatment period.

A long-term treatment with Lipostabil was carried out in Japan by Funatzu (1966). Twenty-nine patients with arteriosclerosis and hypertension were given an oral daily dose of 1,050 mg Lipostabil for the first six months and half of that amount daily over a period of several years. In 19 subjects the cholesterol level fell significantly, i.e., by 35 mg cholesterol/100 ml serum or more; in the remaining 10, the change was not definite. In most cases, the optimal

fall in cholesterol level (∿ 35 mg) was reached after 4 to 8 months of regular treatment. In addition, the cholesterol/phospholipid quotient changed from 1.26 at the start of treatment to 0.95 after 12 to 14 months' treatment.

Contrary to the forementioned studies, the beneficial effects of lecithin could not be confirmed by some investigators. A large trial was carried on a total of 114 subjects suffering from atherosclerotic arterial occlusion. The study lasted for periods up to two years, and employed a double-blind technique (Enticknap, 1962). The dose of Lipostabil ranged from 600 mg to 1,800 mg a day with no specific dietetic policy. No significant differences between the test and control groups were shown in the parameters, such as total cholesterol, β-liporportein and pre-β-lipoprotein. Similar results were obtained by other investigators (Davies and Murdoch, 1959; Butler et al., 1960; ter Welle et al., 1974).

Most studies thus far carried out were on patients with atherosclerotic complications. An attempt was made to determine the effect of lecithin supplementation in normal population of 31 healthy middle-aged volunteers (Halvorson, 1976; Holden, 1976). The dosage level of soybean lecithin was 7.2g, which is frequently recommended in health food stores and the experimental period lasted up to two months. The proportion of cholesterol ester in the plasma increased from 66% to 67% with the supplement. However, there was no change in the other lipid parameters: total cholesterol, total phospholipid, individual classes of phospholipids, total lipids or triglycerides

in the plasma.

The clinical studies, in general, focus on the behavior of serum cholesterol values. This focus has been criticized by ter Welle et al. (1974) who state that changes in blood cholesterol are inadequate measure of changes in total body cholesterol. A systematic study of parameters such as absorption, synthesis, transport and excretion of cholesterol in clearly defined groups of subjects would be desirable in evaluating the role of lecithin in cholesterol metabolism.

Animal studies

In contrast to the human experiments, animal studies are subject to fewer limitations. A number of experiments using rats, rabbits and baboons have provided insight into the possible mechanisms of lecithin action.

In 1942, Kesten and Silbowitz observed that the feeding of crude soya lecithin to chinchilla rabbits receiving cholesterol lowered plasma cholesterol and diminished the incidence of experimental atherosclerosis. The animals were divided into three groups receiving either no lecithin, 1 g lecithin, or 5 g lecithin daily for a period of four months. The resulting plasma cholesterol was inversely related to the amount of lecithin given.

Friedman and his colleagues (1957) induced two different degrees of hypercholesterolemia in male rabbits by feeding 1% and 3% cholesterol with cottonseed oil for three months. The hypercholesterolemic diets were followed by Purina rabbit chow for a period of 3 months.

Then half of each group received 600 mg animal lecithin infused into the external jugular vein: the other half received saline. This treatment was repeated four times for the less hypercholesteremic and five times for the severely hypercholesteremic groups. The infusion of phosphatide resulted in a temproary elevation of plasma phospholipid and cholesterol. However, a marked resolution of the atherosclerotic infiltration and cholesterol deposits in the previously hypercholesteremic animals was observed. The authors suggested that the infusion of phosphatide led to a mobilization of cholesterol, in part at least from the aortic deposits of cholesterol, as manifested by temporary hypercholesteremia.

The function of lecithin appears to be dependent in part on its fatty acid composition. The effects of saturated and polyunsaturated lecithin on the removal of cholesterol implanted in the subcutaneous tissues of the rats were studied by Adams and Morgan (1967). The respective mixtures, either cholesterol and ovolecithin or cholesterol and Lipostabil, were implanted under the skin of the anterior abdominal wall. After seven days, ovolecithin accelerated the resorption of subcutaneious implants of cholesterol. This effect was more marked when polyunsaturated lecithin was implanted. The authors attributed the dispersing effect of lecithin to the surface-active or detergent properties of the polarized phosphoryl and choline groups; they suggested that the polyunsaturated fatty acids might be responsible for the rapid resorption of cholesterol.

Adams et al. (1967) further investigated the effect of different lecithins on the development of cholesterol-induced fatty liver and atheroma in rabbits. Repeated intravenous injections of ovolecithin diminished fatty liver in cholesterol-fed rabbits, but did not protect the aorta against atheroma. On the other hand, the injection of polyunsaturated lecithin protected cholesterol-fed rabbits from both fatty liver and aortic atheroma. The amount of polyunsaturated fatty acids in lecithin was thought to determine their anti-atherogenic activity.

From the University of Cambridge, many studies were reported on the effect of lecithin injection on aortic enzymes in rabbits and baboons (Patelski et al., 1970; Waligora et al., 1975; Howard et al., 1971; Howard and Patelski, 1974). In one of the early studies (Patelski et al., 1970), male adult rabbits were fed a semi-synthetic diet containing 20% beef tallow, which produced hyperlipemia and atherosclerosis. When either 1 mg Lipostabil for 10 weeks or 0.5 mg Lipostabil for 18 weeks was injected every second day, the plasma cholesterol was not different from that of the groups without Lipostabil treatment. However, reduced severity of atherosclerosis occurred as a result of the treatment. Compared with the rabbits on an atherogenic diet without Lipostabil treatment, higher cholesterol esterase and lower lipase and phosphilipase A in the aortic wall were seen in groups with Lipostabil. Enhanced serum and liver lipase activities were also observed with Lipostabil treatment: an increase in lipolysis in the circulating blood and liver would act in removing triglycerides which

are the main substrate for the arterial lipase. Hence, Lipostabil was considered to deter the accumulation of cholesterol esters in the arterial wall by enhancing hydrolysis of cholesterol esters.

In the subsequent study (Waligora et al., 1975), formation of cholesterol esters was consistently reduced in atherosclerotic rabbits given Lipostabil solution. The diet consisted of 1% cholesterol and 5% corn oil. The increase in serum and liver lipase activities and the decrease in aortic lipase were also observed with a single injection of Lipostabil in 4% sodium deoxycholate. However, the influence of Lipostabil solution on the various enzyme activities was different in different tissues and dependent on the kind of diet and amount of fat given to the animals. Nevertheless, Lipostabil treatment lowered the cholesterol ester synthesis/hydrolysis ratio, whereas decreased cholesterol esterase and ACAT (Acyl-CoA:cholesterol acyltransferase) activities were seen in the aortas of the animals with atherogenic diet only. This result cannot be interpreted as a sole function of Lipostabil solution since the simultaneous effect of sodium taurocholate on enzyme activities cannot be overlooked. However, the overall results were in favor of the antiatherosclerotic effect of Lipostabil solution resulting from reduced net synthesis of cholesterol esters in the aortic wall.

The effect of Lipostabil on reducing atherosclerosis by changes in aortic lipolytic enzymes was again confirmed by the same group using a different animal species (Howard et al., 1971). When treated with 1 g Lipostabil, the hypercholesterolemic baboons with immunologic injury showed a normal aortic lipase and 50% increase in cholesterol

esterase activity in the aortic wall. Similar to the results obtained with rabbits, Lipostabil treatment had no effect on plasma cholesterol, phosphilipids, or the fatty acid composition of the cholesterol esters and PC. In this study, Lipostabil was injected while the animals were on the atherogenic diet.

The possible effect of Lipostabil on the regression of aortic lesions was tested in the baboon (Howard and Patelski, 1974). The animals, previously fed an atherogenic diet for 6 months, were subsequently given Lipostabil solution with the control diet. The animals were treated with the solution thrice weekly for 16 weeks. Lipostabil solution did not produce a regression of aortic lesions but cholesterol esterase was increased in the aorta and heart. The most significant finding was that aortic ACAT was completely inhibited. On the basis of the results obtained with the baboon, the group proposed that the prophylactic effect of Lipostabil solution could be exerted mainly by its inhibition of ACAT rather than by stimulation of cholesterol esterase. In other words, Lipostabil would prevent the accumulation of cholesterol esters in the aorta rather than hydrolyze the cholesterol esters deposited.

In contrast to the findings of the Cambridge group, Samochowiec et al. (1976b) were able to demonstrate a positive effect of EPL on the regression of aortic lesions in miniature pigs. After the animals had been on atherogenic diets for two months, they were given EPL solution with the control diet for two months. The regression of morphological changes was apparent. As was described previously, Friedman et al. (1957) also observed a marked resolution of the

atherosclerotic infiltration and cholesterol deposits when lecithin was injected into rabbits, previously hypercholesteremic. The rabbit had been on the atherogenic diet for 3 months before the treatment began. This discrepancy in findings regarding the regression may be resolved by the experiment of Constantinides et al. (1960). They indicated that early lipid-rich lesions in cholesterol-fed rabbits may regress, but that when fibrosis occurred the lesions were irreversible. Thus, not only the species difference, but also the length of the period the animals are on the atherogenic regime seem to be responsible for the different results obtained.

Thus far, many investigators have made an attempt to explain the mechanism of antiatherosclerotic action of EPL by using different experimental animal models (Rampone, 1973; Saunders and Sillery, 1976). However, the nature of this task is complex. The major problem encountered in this study is the difficulty of interpretation and of direct application to the human situation. Summarizing the studies done on lecithin supplementation, one will find a wide variation in the response to lecithin thereapy among different species. This is understandable because lipid metabolism and atherogenesis differ among the different species.

Cholesterol esterification

Among the possible risk factors, hypercholesterolemia and other forms of hyperlipidemia have been often associated with atherosclerosis (Ross, 1975). It is reported that hypercholesterolemia injures the endothelium, resulting in increased vessel permeability, thus allowing

easier entry of lipid and of low-density lipoprotein (LDL)(Gresham, 1976).

The atherosclerotic lesion is characterized by the deposition in the intima of considerable quantities of both cholesterol ester and free cholesterol. However, the origin of cholesterol in atherosclerotic plaques and the cause of its accumulation in the diseased vessel is still uncertain. It is believed that cholesterol can be synthesized by arterial intima, but most of the cholesterol in the atherosclerotic intima is derived from the plasma LDL (Smith, 1974). In the cholesterol-fed squirrel monkey there was a significant positive correlation between average total plasma cholesterol concentration and cholesterol concentration of both the thoracic aorta and the abdominal aorta (Raymond et al., 1976). However, the fatty acid composition of lipid in the atherosclerotic intima differs markedly from that of serum (Swell et al. 1960a). Numerous enzyme systems responsible for esterification and hydrolsis of cholesterol have been reported (Abdulla et al., 1969; Proudlock et al., 1973; Patelski et al., 1968).

The significance of plasma cholesterol cannot be overlooked because it is known to infiltrate the aortic intima and inner media (Bondjers and Bjorkerud, 1975). But not much is known about the control of the plasma cholesterol concentration (Byers and Friedman, 1956; Bitman et al., 1976; Massaro and Zilversmit, 1977). There are two forms of cholesterol in the plasma: free and esterified cholesterol. An intriguing fact is the relatively constant ratio of free to esterified cholesterol (approximately 1:3) in the plasma in various

animal species (Eggen, 1976; Miller and Thompson, 1973). The free cholesterol, which is a necessary component of biologic membranes, exchanges readily with cholesterol in tissue pools. Cholesterol ester is thought to represent a transport form by which cholesterol could be brought to the liver for further metabolism (Stokke, 1973). In guinea pigs, the injected ^{3}H-cholesterol ester in the very low-density lipoprotein (VLDL) fraction was rapidly cleared from the plasma; 24% was in the liver in 5 min. and 54% in 15 min. (Barter et al., 1977). A minor fraction was shown to be transferred to the LDL. The liver plays a major role in the overall metabolism of cholesterol, however, there is no indication of the presence of any mechanism for transport of a specific amount of plasma cholesterol into liver (Sodhi, 1974; Stokke, 1973). Cholesterol turnover encompasses the processes of absorption, synthesis, transport, and excretion (Corey et al., 1976). The study of cholesterol esterification in the plasma would yield some insight into the regulation of cholesterol turnover.

The main enzymes involved in the formation of cholesterol ester are acyl-CoA:cholesterol acyltransferase (ACAT; E.C.2.3.1.26), cholesterol ester hydrolase (E.C.3.1.1.13), and lecithin:cholesterol acyltransferase (LCAT; E.C.2.3.1.43)(Norum, 1974).

ACAT is found in many tissues from different animals. Hepatic ACAT is present in several species, but not in the human; there is very little, if any, in the guinea pig. This ACAT was shown to have a role in the formation of some of the plasma cholesterol ester in rats (Stokke, 1974).

Cholesterol esterase is found in many tissues, such as liver, pancreas, adrenal gland, gonads and intestine. It acts both on esterification and hydrolysis depending on the conditions. During absorption, the soluble enzyme in the intestinal mucosa catalyzes the extensive esterification of cholesterol in the presence of free fatty acids and bile salts. The resulting chylomicron is transported to the liver and rapidly hydrolyzed (Glomset, 1968).

LCAT is the enzyme which promotes the transfer of a fatty acid from the β-position of lecithin to the 3-hydroxyl group of free cholesterol, the products being cholesterol ester and lysolecithin (LPC)(Glomset, 1968). Most of the fatty acids that the enzyme transfers to cholesterol are polyunsaturated. LCAT acts primarily on cholesterol in the high density lipoprotein (HDL) and, to a lesser extent, in the low density lipoprotein. Miller and Miller (1975) support the proposition of Glomset (1968) that the transport of cholesterol ester to the liver for subsequent catabolism is the function of the HDL. It has been repeatedly suggested that in humans, most of the plasma cholesterol esters are synthesized within the plasma itself (Stokke, 1973). This notion has been supported by the following observations: 1. for as long as 40 hours after the injection of labeled mevalonic acid the specific activity of esterified cholesterol of the liver was well below that in the plasma (Barter, 1974a), 2. a very low concentration of cholesterol esters is found in the plasma of subjects with familial LCAT deficiency, 3. the esterification of cholesterol by LCAT in vitro shows a similar range of values to that seen in vivo (Clifton-Bligh et al., 1974), and 4. the fatty

acid composition of the cholesterol esters fomred by LCAT reaction is virtually identical to that of the cholesterol esters in plasma.

The occurrence of hereditary LCAT deficiency prompted the study of LCAT and convinced many of the importance of this enzyme (Gjöne, 1974). The characteristics of the plasma lipids in this disease include: 1. high PC and low LPC, 2. less than 10% of cholesterol as cholesterol ester, 3. cholesterol ester composition similar to that of chylomicrons in thoracic duct lymph, 4. minimal in vivo esterification of labeled cholesterol following the infusion of mevalonic acid-^{14}C, and 5. elevated triglycerides. These lipid abnormalities are considered to be secondary to a deficiency of LCAT.

Two concepts prevail as to the role of LCAT in the matabolism of lipoproteins and in the transport of serum lipids: LCAT as part of a removal mechanism for excess cholesterol and as a participant in the removal of triglycerides in conjunction with lipoprotein lipase.

With regard to cholesterol metabolism, it is postulated that unesterified cholesterol in the peripheral tissues diffuses into the lymph and the blood stream where it is esterified upon contact with LCAT and HDL. The cholesterol esters are then transported to the liver where they are hydrolyzed and excreted. Clifton-Bligh et al. (1974) measured plasma LCAT along with total cholesterol turnover in hypercholesterolemic patients before and after colestipol resin therapy and found a similar order of magnitude to the values. Thus, they suggested that most cholesterol molecules are probably esterified within the plasma at some stage during their passage from the sites of synthesis to those of excretion and catabolism. However, during

the control period, the rate of cholesterol esterification was greater than the turnover rate of total body cholesterol, indicating a recycling of cholesterol through plasma after the hydrolysis of cholesterol esters within body tissues. In this study, increased cholesterol esterification was accompanied by a small but significant increase in percent of free cholesterol, contrary to expectation. The notion that the rate of cholesterol esterification appears to be regulated according to changes in the metabolism of cholesterol was supported by Takeuchi and Yamamura (1973) who observed accelerated cholesterol esterification in rats with biliary drainage.

Nestel et al. (1974) suggested that cholesterol ester turnover participates in the increased turnover of triglycerides. Nichols and Smith (1965) have demonstrated a transfer of esterified cholesterol from HDL to VLDL in exchange for triglycerides. This in vitro exchange occurred most repidly with hypertriglyceridemic serum, thus lending further support for an association between cholesterol esterification and triglyceride transport. However, Barter (1974b) could not support a simple precursor-product relationship between the pools of esterified cholesterol in HDL and VLDL of normolipidemic subjects.

Thus far, the factors regulating cholesterol esterification are not fully understood. Information is available on the effect of age (Gherondach, 1963), sex (Lacko et al., 1974; Wallentin and Vikrot, 1975), vitamin deficiency (Wells and Hogan, 1968) and of hormones (Wells, 1971). The observation that diminished efficiency of esterification of cholesterol occurred in coronary heart disease (Soloff et al., 1973) raises the possibility that LCAT activity may

also serve to retard the effect of forces that favor intra-arterial accumulation of cholesterol and the development of atherosclerosis. Furthermore, it suggests the possibility of using LCAT for a screening procedure to uncover subjects prone to develop atherosclerosis and myocardial infarction. However, considerable difficulties are encountered in the interpretation of the results due to the different methods adopted for the measurement of esterification (Shapiro et al., 1968; Glomset, 1969; Stokke and Norum, 1971), use of different experimental models, and the paucity of the studies done on the subject.

Recently, many investigators have made an attempt to explain the mode of action of lecithin supplementation on cholesterol esterification. In Simon Stevin Research Institute in Belgium, soybean lecithin was administered intravenously to 100 patients with type II hyperlipoproteinemia (Medical World News, 1974). The marked reduction of plasma cholesterol was accompanied by a 3% increase in linoleic acid in cholesterol ester. It was postulated that polyunsaturated PC was a more active cofactor for cholesterol esterification, thus resulting in a faster removal of plasma cholesterol. This finding is also supported in rats. Takeuchi and Yamamura (1973) observed markedly enhanced esterification in serum of the rats fed 1% lecithin. In addition, in vitro addition of lecithin to serum increased esterification.

The change in the ratio of free to esterified cholesterol in serum as a result of lecithin supplementation has seldom been reported. The available data are rather inconsistent; no change (Blaton et al.,

1972), increase (Skorepa et al., 1974; Konecki et al., 1962), and decrease in the ratio (Holden, 1976) have all been reported.

At the present time, the significance of plasma esters and the factors regulating esterification are not fully understood. A clarification of the role of cholesterol esters would aid in understanding the mechanism underlying overall cholesterol metabolism.

Metabolism of PC and LPC

In the blood, phospholipids play an important role in the structure and in the formation of lipoproteins. However, very little is known on the regulation of the absorption, transport, and the formation of phospholipids in the blood. The present review will narrow its scope to the metabolism and significance of PC and LPC among the classes of phospholipids.

Regarding the absorption of PC, Rodgers et al. (1975) confirmed that luminal LPC, formed by hydrolysis of PC by pancreatic phospholipase, can be absorbed without further digestion. This study was carried out in the rat by perfusing radioactive lecithin intraduodenally. LPC was also utilized for mucosal PC synthesis by an enzyme system in mucosal microsomes. Parthasarathy et al. (1974) also demonstrated the further hydrolysis of PC to glycerylphosphoryl choline (GPC), glycerol phosphate, glycerol and inorganic phosphorus.

There are four mechanisms for the conversion of PC to LPC in the blood: LCAT, plasma phospholipase, post-heparin phospholipase, and hydrolysis by membrane-bound enzymes (Polonovski, 1972). The

action of LCAT has already been described in the text (p. 14). Plasma phospholipases are very unequally distributed in the animal species. There are no directly active phospholipases for the PC of the blood plasma in man, cats, rats and other mammals, although birds have an active enzyme. Post-heparin phospholipase removes the fatty acid from the C-1 position of glycerol of the PC. The identity of this enzyme is quite obscure; some investigators claim that this enzyme is the same as post-heparin lipoprotein lipase. In any case, this enzyme does not function under normal physiological conditions. Another possibility is that similar membrane-bound enzymes can split phospholipids as the circulating blood contacts the cell membranes of all tissues. The hydrolysis of phospholipids under normal conditions is of little significance. If the membranes of erythrocytes and platelets are ruptured, phospholipase activity in the cells and in the supernatant plasma is observed. Phospholipid hydrolysis is also seen during blood coagulation.

In view of the known toxic and lytic properties of LPC, the rapid elimination of the LPC appears mandatory. LPC has been reported to have a high affinity both for plasma lipoproteins and for cellular membranes including plasma membranes (Portman and Illingworth, 1973; Portman and Alexander, 1976). It is postulated that LPC might serve as a prefabricated component of PC of cell membranes in the tissue.

Due to the complexity of analytical methods, there have been few investigations on the plasma phospholipid profiles in relation to disease. Furthermore, the data that are available are contradictory.

Total phospholipid, PC, and LPC were analyzed in normal men and women by Böttiger (1975). The three parameters were found to increase to a significant degree with age. While total phospholipids and PC were the same for men and women, LPC was significantly lower in women (9.2% of total P) than in men (10.3%), absolutely as well as relatively. Estrogen was thought to be responsible for this sex difference. Christian et al. (1964) observed an absolute increase of all fractions except sphingomyelin in essential hypertriglyceridemia and an absolute increase of all fractions except LPC in essential hypercholesterolemia. Kunz (1970) reported a significant elevation of the relative and absolute amounts of PC, PS (phosphatidyl serine), PI (phosphatidyl inositol) and PE (phosphatidyl ethanolamine) at all stages of type IV hyperlipoproteinemia. There was a conspicuous decrease in absolute amount of LPC.

Similar results were documented by Gillett and Besterman (1975) with 77 healthy subjects and 76 patients suffering from atherosclerotic diseases. The most consistent difference in the phospholipid composition of plasma obtained from healthy male subjects and from those suffering from atherosclerotic disease was found in the LPC fraction. The relative and absolute concentrations of plasma LPC were lower in men suffering from chronic ischaemic heart disease and peripheral arterial disease when compared with age-matched healthy male subjects. The lowest level of LPC was associated with acute myocardial infarction. Another finding was that relatively low levels of LPC in erythrocytes and platelets were associated with decreased plasma LPC, which implied

that the LPC content of blood cells may be derived from the plasma LPC pool. Rapid and complete exchange of plasma LPC and PC with erythrocytes has been reported (Tarlov, 1966).

The above observations were also confirmed in other species (Mohan and Chakravarti, 1975). In the rhesus monkey, the lipid profile showed a very marked difference between induced atherosclerosis and spontaneous atherosclerosis. In spontaneous atherosclerosis, plasma PC was significantly elevated, while LPC was diminished; in cholesterol-induced atherosclerosis, plasma PE was elevated and LPC was not different from normal.

The metabolism of LPC in the primates was extensively studied by Portman et al. (1970). As Stein and Stein (1966) had observed in the rat, injected LPC was rapidly taken up into the tissues. The authors argue that the plasma LPC seemed to be part of a much larger pool, and that the total production rate of LPC in this pool was much larger than could be accounted for by the plasma LCAT activity alone. Thus, there is the possibility of an exchange of LPC resulting from tissue pholpholipase activity and tissue LCAT activity. Contrary to spontaneous atherosclerosis, diet-induced atherosclerosis in monkeys results in increased concentrations of LPC and PC in the aorta and plasma. There was an increase in plasma LPC resulting from the increased activity of LCAT. The time for equilibration of plasma and aorta LPC-^{14}C was much greater for the diet-induced atherosclerotic group and the increase in concentration of LPC in the aorta occurred more slowly than that in plasma.

In a recent study, Portman and Alexander (1976) indicated that LPC may influence the removal of lipoproteins from the plasma to tissues by acting as a bond between cellular membranes and plasma lipoproteins. When pieces of aorta from rabbits or rhesus monkeys were preincubated with LPC, and then with labeled lipoproteins, the retention of low, very low, or high density lipoproteins by the intima and inner media increased. A subsequent incubation of the piece of artery in a medium containing trypsin or LPC caused a release of some of the lipoproteins. Intravenous injection of LPC increased the rate of removal of LDL from the plasma of rabbits and squirrel monkeys, but there was no significant effect on the retention of radioactivity by aortic intima plus inner media. This observation was somewhat consistent with that of Dobiásová et al. (1976).

Dobiásová and Faltová (1975) showed that intervention into lecithin metabolism in plasma could induce direct effects on the distribution and transport of plasma cholesterol. The investigators administered phospholipase A to rats. Immediately after the application of phospholipase A, there was an equimolar decline in the plasma concentrations of lipid phosphorus and cholesterol ester. Ten minutes later, there was an increase of lipid phosphorus and total cholesterol in the liver, primarily, and also in the lungs. This study, carried out under a special model situation provided new insights into the role of LPC in cholesterol metabolism.

In further research, Dobiásová et al. (1976) observed a higher clearance rate of ^{14}C-cholesterol when administered with LPC than

when given with PC. An intriguing finding was that the amount of ^{14}C-cholesterol esterification in the plasma was significantly higher when administered with LPC than with PC. This is apparently in contradiction to the finding of Nakagawa and Nishida (1973) who demonstrated an inhibitory effect of LPC on LCAT activity in vitro. Nevertheless, the authors speculated that LPC accelerates the exchange of cholesterol between the organs of the fast pool and possibly increases the accumulation of cholesterol into the slow pool tissues, thus acting as an atherogenic substance.

At the moment, the fragmentary observations create a great confusion on the relation of LPC and PC to atherogenesis. The decrease of the LPC fraction in the plasma of atherosclerotic patients cannot be explained by the atherogenic effect of LPC.

Platelet aggregation

Evidence has accumulated indicating that platelets may play a predominant role in initiating thrombotic complications of atherosclerosis (Poole, 1973). In fully-developed human atherosclerosis, scanning electron microscopy of femoral arteries revealed platelet aggregates and cholesterol crystals on top of atherosclerotic plaques (Hess, 1973).

Platelets play an important function in the hemostatic mechanism. They also provide a source of phospholipoprotein to promote clotting (Broekman et al., 1976). The inert, circulating platelets in normal subjects do not adhere to each other or to normal vascular endothelium.

However, platelets readily adhere to exposed elements of the blood vessel wall, such as collagen, basement membrane and microfibrils associated with elastin (Weiss, 1976). Adhesion initiates a sequence of biochemical and ultrastructural changes known as the release reaction, during which adenosine diphosphate (ADP) and other granule-bound substances are selectively secreted. The release reaction is followed by platelet aggregation, the mechanism of which is still not clear. The subject is thoroughly reviewed by ten Cate (1971).

Many methods for a quantitative determination of platelet aggregation have been developed since Born (1962) expressed platelet aggregability in terms of optical density. These tests include the clot retraction inhibition test (Gaetano et al., 1973), the platelet-count ratio technique (Wu and Hoak, 1974), the filtragometer procedure (Hornstra et al., 1973), a sensitivity test by microscopic method (Yamakido et al., 1970), and a method using a rotor apparatus (Moriarty et al., 1975). Interpretation of the studies done on platelet aggregation is somewhat complicated, since there is no standard method adopted thus far.

In general, significant enhancement of platelet aggregation has been well documented in the following pathogenic conditions: acute thrombosis (Yamazaki et al., 1975), acute myocardial infarction (O'Brien et al., 1966), transient ischemic attacks (Wu and Hoak, 1975), and the type II hyperlipoproteinemia (Carvalho et al., 1974). According to Rosenberg and Sell (1975), platelets can produce occlusive vascular lesions without the contribution of fibrin.

There is general agreement that hyperlipemia is an important contributory factor to platelet aggregation and thrombus formation, although some results are conflicting (Renaud et al., 1970; Renaud and Gautheron, 1975; Sano et al., 1973; Oversohl et al., 1975, Ferguson et al., 1973; Wu et al., 1975; Kumar et al., 1976).

Renaud et al. (1970) compared the effect of different fats in the diet on endotoxin-induced thrombosis. Rats fed a hyperlipemic diet containing butter or stearic acid showed greater susceptibility to in vitro thrombin-induced aggregation than rats fed corn oil. Increased susceptibility to in vitro thrombin-induced aggregation coincided with the production of multiple large thrombi with endotoxin injection. On the other hand, platelets from rats fed the thrombogenic diets were less responsive to ADP and collagen induced aggregation. Similar results were obtained with the washed platelets. Hence, they postulated that some factors in the platelet itself are responsible for the differences obtained, since changes in plasma could not have affected the platelet behavior under these experimental conditions.

The direct effect of hyperlipemia on thrombosis was studied by Kim et al. (1976). A loop shaped polyethylene cannula was inserted into the aorta of the rats to assess the potential for thrombosis. In the congenitally hyperlipidemic rats, this loop was more rapidly obstructed than in the controls; the converse was true of the hypolipemic group. There was a good correlation between the levels of many coagulation factors and serum cholesterol in both rats and monkeys. This study implies the importance of thrombosis in the

genesis of atherosclerosis.

Adopting different methods for measuring platelet aggregation, many investigators have tested the effect of high blood cholesterol on platelet aggregation. Sano et al. (1973) demonstrated an enhancement of ADP-induced platelet aggregation by cholesterol in rabbits. Three hours after oral administration of 1 g/kg of cholesterol in the rabbits, the intensity of aggregation by ADP as well as the sensitivity to ADP aggregation had increased significantly. This increased sensitivity of platelets to aggregating agents, such as ADP, epinephrine, and collagen, was also observed in the type II hyperlipoproteinemic patients (Carvalho et al., 1974). In other words, the minimum effective concentration of aggregating agents required to induce platelet aggregation was much lower in the patients with type II hyperlipoproteinemia.

Oversohl et al. (1975), using a "micro-photometric" method, observed a significantly enhanced platelet aggregation in rabbits treated with a high cholesterol diet (2%). Interestingly, platelet aggregation was most pronounced when stress (swimming or orthostasis) was applied to the hyperlipidemic animals. An increased catecholamine release upon stress was suspected. The enhanced platelet aggregability as a result of interaction of the lipid and stress has further implications in human atherosclerosis.

By a platelet count-ratio technique, Wu et al. (1975) observed increased platelet aggregates only at an early phase but not at a chronic stage of cholesterol-induced atherosclerosis in the rhesus monkey. The authors explain that the disruption of the endothelium

by cholesterol may have rendered available subendothelial tissues to which circulating platelets adhere. However, the normalization of platelet aggregates at the later stage despite a persistent hypercholesterolemia was not clarified. It is conceivable that hypercholesterolemia per se does not account for the occurrence of increased platelet aggregates.

It has been reported that there were fewer thromboembolic episodes in patients on diets rich in polyunsaturated fatty acids than in patients consuming more saturated fats (Food and Nutrition Research, 1974). Using a filter loop technique which assesses platelet aggregation in flowing blood, Hornstra et al. (1973) found a significantly decreased aggregability of platelets in the subject receiving a diet low in saturated fat and high in polyunsaturated fat. Fleischman (1975) also noted the longer aggregation time on a linoleic acid-enriched diet. A significant response was observed in as little as 48-96 hours after the administration of polyunsaturated fat in the diet. Therefore, the time required for this response appeared to be much shorter than that required to elicit changes in the serum lipids. This points up the usefulness of this test of platelet aggregation as an indicator of the effectiveness of dietary treatment for coronary heart patients. The definite beneficial effect of unsaturated fatty acids on reducing the incidence of ischemic heart disease in men has been established (Turpeinen et al., 1968; Miettinen et al., 1972). In contrast, the rate of platelet aggregation with ADP and collagen was increased during linoleic acid

treatment of healthy men in the study by O'Brien et al. (1976). However, all the other measurements, such as platelet retention in the glass-bead column and bleeding time, were in favor of the decreased reactiveness of platelets with the treatment.

There is evidence that changes in plasma LPC may affect platelet behavior. Besterman and Gillett (1971) found that LPC significantly inhibited irreversible aggregation caused by ADP, epinephrine, and collagen, which suggests that the mechanism underlying aggregation is common to all three reagents. Saturated LPC, which is the main component of plasma LPC, was shown to be a more potent inhibitor than unsaturated LPC (Besterman and Gillett, 1972). LPC concentrations required for maximal inhibition were in the range of 0.2 - 0.7mM, which is higher than in normal plasma. The inhibitory action of LPC on platelet aggregation was also shown by inducing the increased formation of LPC in incubated human plasma after intravenous administration of heparin (Besterman and Gillett, 1973). This increased LPC formation after heparin injection was attributed to an enzyme other than LCAT, since the rate of cholesterol esterification in plasma remained stable. The enzyme responsible for the formation of LPC was partly characterized as a lipoprotein lipase, since it was inhibited by protamine sulphate. A reduced rate of irreversible platelet aggregation was also observed in the absence of significant increases in LPC levels in incubated post-heparin plasma. The mechanism responsible for this phenomenon was not clear.

Subsequently, Joist and Mustard (1974) formulated a hypothesis on the effect of heparin on platelet function. They postulated that

heparin may induce the release of phopholipase and lipoprotein lipase from vascular endothelium. This would, in turn, lead to liberation of both LPC and free fatty acids (FFA) from plasma lipoproteins. The resulting LPC and FFA could compete for the binding site on plasma albumin. If the available binding sites on plasma albumin were occupied by LPC and FFA, additional FFA liberated from lipoproteins could accumulate on the platelet membrane, thereby increasing the platelet reactivity to surface or chemical stimuli. Alternatively, saturation of the albumin binding sites by FFA could lead to increased uptake and metabolism by platelets of LPC, resulting in inhibition of platelet aggregation. However, under certain conditions LPC can potentiate platelet aggregation. Joist et al. (1977) observed a transient potentiation of platelet aggregation by LPC when thrombin was used as the stimulus and suspensions of washed platelets in Tyrode solution containing 0.35% albumin were used. The metabolic products of LPC hydrolysis, GPC and FFA, were suspected to be responsible for the potentiation.

At the moment, the significance of LPC with respect to platelet function in vivo remains to be established. However, in view of the reduced concentration of LPC in patients suffering from myocardial infarction (Berlin et al., 1969) and from type IV hyperlipoproteinemia, it is conceivable that the platelets from such patients are more susceptible to aggregation and thrombus formation. Thus far, the effect of lecithin supplementation on the platelet behavior has not been studied extensively.

The guinea pig as an experimental model for the present study

Extensive work has been done on experimental atherosclerosis in various animal species. Unfortunately, some of the results have either no bearing on the human condition or they are believed to have no value in its interpretation (Altschul, 1950a). The prerequisites of a satisfactory animal model in atherosclerotic research were put forward by Clarkson (1963):

1. The regimen to induce atherosclerosis should be physiologically reasonable.
2. The regimen should produce lesions in the aorta, and also in the renal, coronary and cerebral arteries.
3. The aortic lesion should be most severe in the distal rather than the proximal segments.
4. The atherosclerotic lesion should progress from a fatty streak to thrombus formation.
5. The atherosclerotic plaques should lead to partial or total occlusion of the vessel in a reasonable number of animals.

Altschul (1950b) claimed that experimental arteriosclerosis could be produced in guinea pigs without great difficulty. In guinea pigs, the reaction to a high cholesterol diet is less regular, less intense, and produces somewhat different manifestations than are obtained with rabbits. With dried and heated egg yolk, Altschul demonstrated lipoid deposits, some intimal proliferation, and calcified atheroma in coronary arteries. Calcified skeletal muscle and suprarenal cortex were also seen.

This was further supported by Babala and Ginter (1968). A diet containing as little as 0.25% cholesterol in butter produced changes in the coronary vessels of male guinea pigs after 116 days. Initial changes in the coronary arteries consisted of edema of the arterial wall and focal damage to the endothelial cells. Homogeneous masses, having the characteristics of coagulated lipemic plasma and disintegrated thrombocytes were deposited on the wall of the coronary artery and became incorporated therein, beneath a new layer of endothelium. In the intima of the aorta and the main coronary arteries there were foamy histocytes: in the subintimal layer, there was a slight splitting of elastic fibers. The authors suggested a thrombogenic origin for the experimental coronary lesions in guinea pigs. Thus, histologically, guinea pigs appear to be suitable as an experimental model.

The response of guinea pigs to various levels of dietary cholesterol in guinea pigs differ significantly. There is an increased mortality rate with higher doses of cholesterol (Babala and Ginter, 1968). In addition to the vessel changes, the general responses to dietary cholesterol are: depressed growth rate (Matin and Ostwald, 1975), a rapid and extensive expansion of the body cholesterol pool (Ostwald and Shannon, 1964), histopathological changes in the liver, spleen, kidneys and testes, and hemolytic anemia resulting from morphological and functional changes in red blood cells (Yamanaka and Ostwald, 1968; Ostwald et al., 1970; Ostwald et al., 1977; Drevon and Hovig, 1977).

Cholesterol metabolism is subject to different mechanisms of control among several mammalian species. Cholesterol feeding markedly inhibits hepatic cholesterol synthesis in the rat, dog, and squirrel monkey, but only slightly in the guinea pig (Turley and West, 1976). The rate of cholesterogenesis in the ileum is about four times that in the liver in humans, and about 10 times in guinea pigs, whereas a relatively lower ratio is observed in the rat and monkey. The relative rates of sterol synthesis in the guinea pig suggest that this species may show similar tissue differences in the rate of sterol synthesis to those in the human. The feeding of 5% cholesterol resulted in an active cholesterol feedback system in all tissues of the guinea pig including lung, ileum, and brain (Swann et al., 1975; Turley and West, 1976).

When the guinea pigs were fed a diet containing 0.1% cholesterol for 15 to 19 weeks, a significant increase in the cholesterol concentration of red blood cells, liver and the wall of the small intestine resulted (Green et al., 1976). The cholesterol concentration of spleen, lungs, heart, kidneys and carcass remained unchanged and hypercholesterolemia did not develop. The percent of tracer cholesterol absorbed was the same in the control guinea pigs (53%) as in those fed the diet containing 0.1% cholesterol (52%). On the other hand, the output rate for the cholesterol-fed animals was 42% greater than for the controls, which might imply an increased bile acid excretion. In humans, cholesterol feeding appeared to have little influence on endogenous synthesis but did produce an increase in the excretion of cholesterol (Nestel and Poyser, 1976), although marked variability

among individuals existed.

Almost no hepatic ACAT activity has been detected in guinea pigs or humans, whereas significant activity was observed in dogs, swine, rats and monkeys (Stokke, 1974). Therefore, plasma LCAT activity is probably a major factor in esterification of plasma cholesterol, in the guinea pig as in the human. However, esterification by intestinal mucosa cannot be overlooked. The fatty acid composition of cholesterol esters in the plasma of guinea pigs was shown to be similar to that of humans in which linoleic acid is a predominant fatty acid (Swell et al., 1960b).

The aggregation of platelets of different animals, in response to different aggregating agents, seems to be an extremely variable phenomenon. Mills (1970) claims that none of the animals so far examined is a very good model for the behavior of human platelets. In some respects, the platelets of baboons, cats and guinea pigs resemble those of humans more closely than do those of rabbits, rats, dogs and the larger domestic animals. Mills and Thomas (1969) compared the ATP and ADP levels in platelets of man and other species. Absolute amounts of ATP were almost the same in humans, rabbits, guinea pigs and rats. However, significant differences in the ADP concentrations of the different species resulted in ATP/ADP ratios of 1.76 in humans, 2.76 in guinea pigs, 3.76 in rats, and 6.66 in rabbits.

Guinea pig platelets are more responsive to the aggregating action of ADP than are those of man (Constantine, 1966). Platelet aggregation was proportional to the concentration of ADP, and

adenosine did not inhibit ADP-induced aggregation. Epinephrine and 5-hydroxytryptamine (5-HT), both at 10^{-5}M, did not cause aggregation.

On the basis of the foregoing considerations, it would be reasonable to assume that guinea pigs will be a suitable animal model for the present experiment.

III. MATERIALS AND METHODS

Experimental design

The design of the experiment is summarized as follows:

1. Experimental animal: Male adult guinea pigs, 12 animals per group
2. Experimental groups:
   A. Control: powdered guinea pig chow + 10% fat
   B. Lecithin: control diet + 1% lecithin
   C. Cholesterol: control diet + 0.25% cholesterol
   D. Cholesterol + lecithin: control diet + 0.25% cholesterol + 1% lecithin
3. Experimental period: Initial adjustment period of 1 week each on guinea pig pellet and powdered high-fat diet, followed by 8 weeks on the respective experimental diet
4. Parameters measured:
   - Food intake
   - Body weight
   - Plasma components:
     Cholesterol - total, free, esterified
     Phospholipids - total, PC, LPC
     LCAT activity
     in vitro platelet aggregation
   - Tissue cholesterol: liver, kidney, lung, spleen, digestive tract, and carcass

Diets

The composition of each of the four diets is shown in Table 1. Pelleted guinea pig chow was ground with a Waring Blender. For the diets containing cholesterol (C & D), cholesterol was dissolved in the coconut oil and heated at 110°C for 10 min. After the oil was cooled to room temperature, the ingredients specified in Table 1 were mixed in a Hobart Commercial Mixer at speed 2 for 30 min. The diets were kept refrigerated.

Animals

Male adult, Hartley guinea pigs, weighing 700-950 g, were obtained from Camm Research Institute, Inc. (Wayne, N. J.). At the time of shipment, they were 19 weeks old. The guinea pigs were housed singly in screen-bottomed stainless steel cages in an air-conditioned room. Lighting was regulated automatically to provide 12 hours of light (7:00 a.m. to 7:00 p.m.) and 12 hours of darkness. Fresh water containing 0.002% ascorbic acid was supplied daily.

Upon arrival, the guinea pigs were quarantined for 48 hours. For the first week, they were fed pelleted guinea pig chow. During the second week, they received a powdered stock diet containing 10% coconut oil for a week to get them used to a powdered, high-fat diet. They were then randomly assigned to groups.

Animals were fed the respective diets ad libitum. Food consumption was measured every other day and the body weights were recorded weekly.

Table 1. Diet composition (percent by weight)

Diet	Coconut oil[a]	Guinea pig chow[b]	Soy Lecithin[c]	Cholesterol[d]
A. Control	10	90	-	-
B. Lecithin	10	89	1	-
C. Cholesterol	10	89.75	-	0.25
D. Cholesterol+ Lecithin	10	88.75	1	0.25

[a]Gift of PALMCO, Portland, Oregon

[b]Ralston Purina Co., St. Louis, Mo.

[c]ICN, Irvine, Calif.

[d]Eastman, Rochester, N. Y.

Blood was drawn by heart puncture at 0, 2, 5, and 8 week intervals after the iniation of experimental diet. At the end of 8 weeks, animals were killed by CO_2 inhalation. Liver, kidneys, lungs, and spleen were excised, then blotted with the filter paper. The digestive tract except esophagus was slit open and the contents were rinsed out and blotted with filter paper. The organs were weighed and frozen until further analysis. The remaining carcass was rinsed out and frozen. The thoracic aorta was incised longitudianlly from its origin to its upper abdominal part, freed of excess adventitia and fixed in 10% neutral buffered formalin. The heart was also kept in 10% neutral buffered formalin.

Blood drawing and RBC counting

Two animals from each experimental group were subjected to blood drawing each day. Prior to the blood drawing, the animals were fasted for 10 hours. The guinea pigs were quickly anaesthesized in a dry ice chamber (2 lbs of 2 inch cubes) (Hyde, 1962; Hoar, 1969), and tied to the restraining board. The section around the heart was sterilized with 70% ethanol. Heart puncture was done while the animal was under light anaesthesia with ether by nose cone.

A 10-ml Boyd-Dickinson plastic syring was rinsed with 3.8% trisodium citrate solution, and the blood was drawn into the syringe containing 3.8% trisodium citrate (1 volume of citrate to 9 volumes of blood). After gentle inversion, 3.5 ml blood were transferred to a plastic tube and kept at room temperature for the platelet aggregation test. The remaining blood was transferred into another

tube and kept in ice until centrifuged. As much blood as possible was removed by heart puncture at the time of killing.

Erythrocytes were counted in blood drawn by heart puncture. The blood was diluted with Hayem's solution and counted in a Spencer Bright-Line counting chamber.

Determination and separation of cholesterol

Cholesterol was determined with a Technicon AutoAnalyzer[1] by a modification of the method by Block et al. (1966). Esterified cholesterol and free cholesterol were separated on silicic acid columns as described by Holden (1976). Concentrations of cholesterol are reported in mg/100 ml plasma.

Determination of total phospholipid, LPC and PC

Individual phospholipids were separated by thin layer chromatography, as described by Halvorson (1976). Lipid phosphorus was determined colorimetrically as the molybdenum blue complex, essentially according to the procedure outlined by Halvorson. The only deviation from that was the ashing step. The lipid extract was ashed with 27% perchloric acid in 5N sulfuric acid. After drying at 95°C for 2 hours, the samples were transferred to a 165°C oven and kept for 1 hour. The temperature was raised to 185°C after the end of 1 hour, and 2 more hours were allowed for complete ashing. Acid washing of the tubes and the glassware was necessary. Phospholipids

[1]Technicon Instruments Corp., Ardsley (Chauncey), N. Y.

are reported as mg/100 ml plasma.

LCAT determination

The method of Stokke and Norum (1971) was used. LCAT acts on the native lipoproteins in autologous serum in this assay. Cholesterol esterification is measured as the increase in concentration of esterified cholesterol during incubation of the serum.

The labeled cholesterol-albumin substrate emulsion was prepared immediately before the assay. In order to obtain a clear emulsion, it was necessary to evaporate the acetone completely under a stream of nitrogen. The labeled cholesterol substrate was equilibrated with plasma free cholesterol for 4 hours, during which time plasma LCAT activity was inhibited by 1.4 mM Ellman reagent (5,5-dithiobis (2-nitrobenzoic acid)). The enzyme was reactivated by 0.1 M mercapto-ethanol and the activated samples were incubated at 37°C. Duplicate samples and one blank, in which enzyme was not reactivated by mercaptoethanol, were analysed for each sample.

After one hour of incubation, the reaction was stopped by the addition of isopropanol. Cholesterol ester and free cholesterol were separated by column chromatography as described previously. The resulting eluates were dried under nitrogen, 10 ml scintillation solvent were added and counted in a Beckman Liquid Scintillation Counter Model LS 3.133P[1]. The aliquots taken before the separation by column chromatography were counted to check the recovery from column.

[1]Beckman Instruments, Inc., Irvine, Calif.

Counting was done twice and the numbers were averaged.

Enzyme activity is expressed in two different ways, as the percent esterification of cholesterol, and as the net esterification (percent esterification x concentration of free cholesterol).

Tissue preparation for cholesterol determination

Individual organs were thawed and chopped finely with scissors. They were then homogenized in methanol with the Micro Virtis Model 45[1]. The homogenate was quantitatively transferred, with methanol rinses, to a measuring cylinder. After complete mixing, aliquots were transferred to a stoppered centrifuge tube. Two volumes of chloroform were added and the lipid extraction was performed according to Folch et al. (1957). The resulting chloroform extract was evaporated to dryness and was subjected to cholesterol analysis on the AutoAnalyzer.

Carcass preparation for cholesterol determination

The carcass was shaved with the animal clipper and was autoclaved for 2 hours (15 lbs pressure, 120°C). All bones were removed. The carcass was blended in a Osterizer[2] for 5 min. at low speed. The resulting homogenate was strained through a plastic strainer. The residues were washed with warm water, and strained: this step was repeated three times. The resulting filerates were pooled and

[1]Virtis Company, Inc., Gardiner, N. Y.

[2]Oster Corp., Milwaukee, Wis.

transferred quantitatively to a graduated cylinder. The final volume was checked and the contents were mixed thoroughly by inversion. Ten to fifteen percent aliquots were stored frozen until further analysis for cholesterol. The procedures for lipid extraction and cholesterol determination were identical to those for the tissue samples.

Platelet aggregation

Platelet aggregation was measured according to the method of Born (1962). The citrated blood was centrifuged at 150 x g at room temperature for 10 min. If the portion of platelet-rich plasma (PRP) was less than 1/3 of the total blood volume, it was recentrifuged at 200 x g for 3 min. The PRP portion was transferred into a clean plastic tube with a siliconized Pasteur pipette. The remaining blood specimen was centrifuged at 1,500 x g to prepare platelet-poor plasma (PPP).

The aggregometer used was the chrono-log Model 330 Platelet Aggregometer[1] and the recorder was a Bausch & Lomb Laboratory Recorder V.O.M.-5[2]. A full scale span of 10mV was used for the recorder. The chart speed was 1 inch/min.

The siliconized 0.312" cuvette containing 0.4 ml PPP was inserted and the PPP baseline was adjusted to 1 on the recorder. Four tenth ml PRP was pipetted into the cuvette and a stir bar was added. The PRP baseline (0% aggregation) was set at 9 on the recorder.

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[1] Chrono-Log Corp., Havertown, PA.

[2] Bausch & Lomb Inc., Rochester, N. Y.

After the PRP baseline had been established for 1 min, 25 µl ADP solution were added and the change in the light transmittance was recorded for 5 min.

The stock ADP solution (1.7×10^{-3}M in imidazole-buffered saline) was stored at - 20°C. Aliquots were thawed immediately before use. Two final concentrations of ADP, 5×10^{-6}M and 3×10^{-7}M, were used in this test. All tests were done within three hours after blood drawing. The intensity of ADP-induced platelet aggregation was obtained as follows:

$$\frac{\text{Maximum deflection of PRP induced by ADP}}{\text{PRP baseline - PPP baseline}} \times 100$$

Analysis of lecithin for fatty acid composition

The refined soy lecithin (acetone insolubles 95%, FFA 1%, ash 9.0%, moisture 1%, N 1%, P 3.3%) was analyzed for its fatty acid composition by gas liquid chromatography (Lowry & Tinsley, 1975; DeMort et al. 1972). Approximately 50 mg lecithin were placed in a screw cap culture tube, and 3 ml ether + 3 ml 5% HCL gas in superdry methanol were added. It was capped and heated at 80°C in a heating block for 90 min. At the end of the heating period, 3 ml of water and 3 ml of hexane were added: the tube was then capped, shaken vigorously and centrifuged briefly. The sample was extracted again with 3 ml of hexane: ether (1:1). One half µl of the extract was chromatographed on an EGS column (ethyleneglycolsuccinate; 200 ft., 0.03" I.D.). The relative fatty acid composition of lecithin

is presented in Table 2. The ratio of saturated fatty acid to unsaturated fatty acid is approximately 1:3.

Data analysis

One way analysis of variance was done to compare the means of various determinations among the experimental groups at each time period. The following groups were compared: control with lecithin, cholesterol with cholesterol + lecithin, and non-cholesterol groups (A+B) with cholesterol-fed groups (C+D). The paired t-test was carried out to compare the means between any time periods within each group. For some independent variables of interest, correlation coefficients were determined.

Table 2. The fatty acid composition of refined soy lecithin[a,b]

Fatty acids	% total
Palmitic acid	21.07
Stearic acid	4.57
Oleic acid	6.60
Linoleic acid	59.80
Linolenic acid	7.94
Arachidonic acid	negligible

[a]Lecithin-soy-refined, ICN, Irvine, Calif.

[b]analysis carried out by Mr. Bob Lowry

IV. RESULTS AND DISCUSSION

In the beginning of the experiment, excess animals were assigned to each group to allow for losses in the course of experiment. As was expected, several deaths occurred during the experimental period due to either pneumonia[1] or trauma from cardiac puncture. All surviving animals appeared healthy. Red cell counts were performed at each time of blood drawing and all animals showed normal counts regardless of dietary treatment. It has been reported that 1% cholesterol feeding to guinea pigs induces hemolytic anemia between the 5th and 7th week (Ostwald et al., 1977). In the present study, no hemolytic anemia was observed in guinea pigs receiving diets containing 0.25% cholesterol (0.53 mg/Kcal).

Food consumption and body weight gain

The data on cumulative food intake over the 8-week period are presented in Table 3. The animals adapted well to the powdered, high-fat diets. The mean total intake of the lecithin-fed guinea pig (Diet B) did not differ significantly from that of the control (Diet A). Similarly, food consumption did not differ significantly between the animals on cholesterol + lecithin (Diet D) and those on cholesterol only (Diet C). However, the mean total food intake of the cholesterol-fed groups, with and without lecithin, was significantly lower than that of the groups receiving no cholesterol

[1] Autopsy carried out by Dr. N. Patton in the Laboratory of Animal Resources at Oregon State University.

Table 3. Cumulative food consumption, weight gain, and feed efficiency in guinea pigs treated with dietary lecithin and/or cholesterol (means and standard deviations).

Group	Food intake - gm			Weight gain - gm			Feed efficiency gm gained/gm eaten
	Week 2	Week 5	Week 8	Week 2	Week 5	Week 8	
Control (A)	481.5 ±82.5	1240.9 ±162.4	1969.0 ±223.9	11.4 ±49.6	58.9 ±55.0	99.1 ±64.4	0.0482 ±0.0275
Lecithin (B)	469.0 ±68.8	1196.0 ±122.1	1878.0 ±169.4	28.1 ±51.1	73.9 ±54.4	90.8 ±44.2	0.0474 ±0.0216
Cholesterol (C)	444.0 ±64.5	1101.0 ±127.4	1761.0 ±203.9	21.1 ±61.3	-3.5 ±53.6	0 ±54.3	-0.0024 ±0.0326
Cholesterol + Lecithin (D)	420.0 ±56.3	1041.0 ±111.5	1674.0 ±156.7	-15.8 ±52.0	5.7 ±50.4	4.9 ±72.7	0.0019 ±0.0437
Statistical comparison (A+B vs C+D)	$p<0.05$	ns	$p<0.05$	$p<0.05$	$p<0.001$	$p<0.001$	$p<0.001$

($p<0.05$). This finding does not agree with the observation by Turley and West (1976) that the food consumption of guinea pigs fed 0.24% cholesterol for two weeks did not differ from that of animals fed the control diet. On the other hand, Matin and Ostwald (1975) found that a diet containing 1% cholesterol significantly lowered the food intake on the second day of feeding and thereafter.

The cumulative weight gain is also shown in Table 3. Animals from both the control and lecithin groups showed normal growth patterns. On the other hand, cholesterol-treated groups, regardless of lecithin supplementation, gained little weight throughout the experimental period. Rather, they maintained the initial weight with fluctuations. Differences in mean weight gain between the groups with and without cholesterol in the diet (C+D vs A+B) attained statistical significance as early as the second week and were highly significant ($p<0.001$) by the fifth week. This failure to gain weight has been seen in guinea pigs treated with 1% cholesterol (Matin and Ostwald, 1975) but not in animals receiving 0.25% cholesterol for as long as 116 days (Babala and Ginter, 1968). The discrepancy in the results must be attributed to the differences in the strain as well as the ages of animals used.

When the ratio of grams body weight gained per gram food consumed is used to measure feed efficiency (Table 3), mean values are consinstently lower for the cholesterol-fed groups, with or without lecithin ($p<0.001$). Although this approach for calculating the feed efficiency is open to criticism, because live weight does not give a correct measure of energy balance, it will provide a rough

estimate of feed efficiency. By contrast, Matin and Ostwald (1975) demonstrated that feeding cholesterol to guinea pigs affects the food consumption but not food utilization. This discrepancy between the two studies can be attributed to the differences in the cholesterol levels in the diets as well as in the ages of animals used. The latter investigators treated young, growing animals with 1% cholesterol, while adult animals and a diet containing only 0.25% cholesterol were used in the present study.

Overall, the present data on growth and food consumption are consistent with the concept that cholesterol feeding is associated with a decrease in body weight gain in guinea pigs. The failure to gain was associated, not only with decreased food consumption, but also with decreased feed efficiency. Lecithin supplementation to the diets, with or without cholesterol, did not exert any influence either on weight or on food consumption.

Concentration of lipid components and activity of LCAT enzyme in plasma

The time course of the responses of plasma lipids to lecithin and cholesterol treatment was studied in relation to LCAT (lecithin: cholesterol acyltransferase) activity. The parameters measured were: total, free, and esterified cholesterol: total phospholipids: PC (phosphatidyl choline) and LPC (lysophosphatidyl choline); and LCAT activity. Cholesterol and PC are substrates for LCAT action whereas cholesterol ester and LPC are the products.

Cholesterol

Table 4 summarizes the data on plasma cholesterol concentrations. Prior to the experimental treatment, the mean concentrations of total cholesterol were comparable to those reported in the literature (Green et al., 1976) and did not differ among the groups. In both the control and the lecithin groups there were no systematic changes in total plasma cholesterol with the duration of the respective diets up to five weeks. However, a significant increase ($p<0.05$) in the total cholesterol concentration over the initial level was seen in control guinea pigs by the 8th week. This can be attributed to the prolonged ingestion of saturated fat (Corey et al., 1976), as well as the stress from the repeated cardiac puncture. Lecithin supplementation to the control diet did not alter the plasma cholesterol level, which agrees well with the observation by Holden (1976) who investigated the effect of soy lecithin treatment in healthy, middle-aged men. Thus, dietary lecithin in the normal animal does not appear to alter the plasma concentration of total cholesterol.

With the atherogenic diet (C), which supplied 0.25% cholesterol, the plasma cholesterol increased rapidly and steeply within two weeks, after which the rate of increase was more gradual. The increase in plasma cholesterol level was statistically significant ($p<0.01$) and was 3-4 times that found in control animals. The wide inter-subject variability in response of plasma cholesterol concentration to an atherogenic diet is evidenced by the large standard deviations. This is in accordance with observations on rhesus monkeys by Eggen

Table 4. Total cholesterol and % cholesterol ester in plasma of guinea pigs treated with dietary lecithin and/or cholesterol (means and standard deviations)

Group	Total cholesterol (mg/100 ml)				% esterified cholesterol			
	Initial	Week 2	Week 5	Week 8	Initial	Week 2	Week 5	Week 8
Control (A)	52.3 ±20.2	57.0 ±16.1	55.4 ±10.6	62.8[a] ±11.2	78.5 ± 4.0	77.6 ± 4.4	78.7 ± 5.3	76.8 ± 4.6
Lecithin (B)	52.5 ±12.3	57.3 ±15.9	56.7 ±14.8	61.9 ±20.5	77.8 ± 3.4	77.5 ± 2.0	77.8 ± 2.0	77.7 ± 3.8
Cholesterol (C)	57.6 ±10.7	190.2[b] ±97.3	228.0[b] ±99.0	241.6[b] ±124.9	78.1 ± 2.5	68.2[b] ± 5.2	68.0[b] ± 2.3	66.5[b] ± 3.1
Cholesterol + Lecithin (D)	52.1 ±11.7	244.2[b] ± 95.0	231.0[b] ±102.5	265.4[b] ±101.9	79.0 ± 3.0	68.7[b] ± 3.4	66.7[b] ± 2.8	67.3[b] ± 2.2
Statistical comparison (A+B vs C+D)	ns	p<0.001	p<0.001	p<0.001	ns	p<0.001	p<0.001	p<0.001

[a]Significantly different from the initial value, p<0.05

[b]Significantly different from the initial value, p<0.01

(1976). Some of the variation in concentration of plasma cholesterol may be accounted for by differences in absorption of luminal cholesterol. In addition, differences in genetic factors, the rate of endogenous synthesis, the fractional removal of circulating cholesterol (Corey et al., 1976), and the disposal rate (Massaro and Zilversmit, 1977) may have caused the marked variability observed. Despite the variability, the plasma response to the cholesterol-containing diets C+D was significantly greater than to the non-cholesterol diets A+B ($p<0.001$).

Since the main interest in lecithin supplementation is focused on the improvement of pathologic conditions, the effect of lecithin added to the atherogenic diet was also observed. An unexpected increase in plasma cholesterol was seen after 2 weeks of lecithin supplementation to the atherogenic diet. The increase exceeded that of the cholesterol group but the difference between the two groups did not attain statistical significance due to the large variability among individual animals. The pattern of increase in plasma cholesterol with time was similar in both groups. Friedman et al. (1957) observed a temporary hypercholesteremia following phosphatide infusion in the rabbit and attributed it to the withdrawal of cholesterol from tissues into plasma. It is unlikely that this explanation can be applied to the findings in the present study because as shown in a later section, no decrease in tissue cholesterol was observed with lecithin supplementation.

Most investigations have revealed a decrease in plasma cholesterol

when lecithin was added to an atherogenic diet (Samochowiec et al., 1976a & b). The investigators frequently ascribe the cholesterol-lowering effect of lecithin to its content of unsaturated fatty acids. The change in fatty acids of cholesterol ester as well as PC, as a result of feeding polyunsaturated lecithin, is thought to be responsible for a more rapid disposal rate of cholesterol from plasma. Changes in the fatty acid fractions of the plasma lipids were not examined in the present study. However, the lecithin supplement contained linoleic acid in a proportion comparable to that of EPL, which was used in the other studies. Moreover, the failure to confirm the reported hypocholesteremic effect of lecithin cannot be explained simply on the basis of differences in dosage level, mode of administration, or treatment period. It is possible that species differences may account for the confliciting results. The findings of the present study are, rather, in agreement with those of the Cambridge group (Howard et al., 1971; Howard and Patelski, 1974), who failed to demonstrate a plasma cholesterol-lowering effect of polyunsaturated PC either in baboons or in rabbits. It appears that the beneficial effect of lecithin supplementation, if it exists, is not mediated by a lowering of plasma cholesterol under these experimental conditions. Patelski et al. (1971) are of the opinion that polyunsaturated lecithin more likely functions to change the aortic enzyme activities, although the mechanism of action remains to be elucidated.

The esterified cholesterol, as percentage of total cholesterol in plasma, is also presented in Table 4. Initially, the mean percentage of cholesterol ester ranged from 77.8 to 79.0%; this is in

agreement with the published report of Green et al. (1976). It remained steady throughout the 8 weeks for animals on the control and lecithin diets (A,B). The reported changes in the proportion of esterified cholesterol in plasma with lecithin treatment are not in agreement. Holden (1976) observed a small, but significant increase in ester fraction after lecithin supplementation in healthy men. In contrast, Konecki et al. (1962) demonstrated a decreased ester fraction with polyunsaturated PC treatment in the rat. In the present study, lecithin supplementation produced no change in esterified cholesterol.

Within two weeks of cholesterol feeding, with or without lecithin, the ester fraction in the plasma decreased significantly ($p<0.01$) to approximately 68%, and continued to decrease slightly for the remaining weeks. Thus, the large increase of total cholesterol was mainly due to the relatively large rise in the free cholesterol fraction. This would suggest a limited ability to esterify cholesterol. Bitman et al. (1976) also observed a decrease in the proportion of cholesterol ester, from 75% to 57-66%, when cholesterol and fat were added to the diet of rabbits.

The addition of lecithin to the atherogenic diet did not lead to a significant change in the proportion of cholesterol ester, compared with the group on cholesterol alone. In contrast, Skorepa et al. (1974) observed that the total cholesterol level, after four weeks of lecithin treatment, fell at the expense of the free cholesterol. They proposed that linoleic acid of PC, not lost by β-oxidation, was utilized in transesterification.

Nonetheless, the lowered fraction of ester in the plasma of the

animals fed the atherogenic diet regardless of lecithin treatment, bears a striking resemblance to the proportions in plasma of coronary patients (Soloff et al., 1974). The importance of this finding will be discussed further in connection to LCAT activity.

Phospholipids

As shown in Table 5, total plasma phospholipid concentrations in both the control and the lecithin groups remained unchanged throughout the 8-week experimental period. On the other hand, with the diets containing cholesterol, there was a significant rise in phospholipid level ($p<0.01$). The increase was slightly higher when lecithin was added simultaneously with cholesterol (D). As with the cholesterol data, wide individual variations occurred. It has been frequently observed that a rise in phospholipids accompanies all hypercholesteremic states (Byers and Friedman, 1956). The published data on the response of serum phospholipids to lecithin treatment are conflicting; no change (ter Welle et al., 1974) as well as a decrease in the concentration (Blaton et al., 1972; Konecki et al., 1962) were observed. Samochowiec et al. (1976b), who observed an elevated level of phospholipids, not only in serum but also in the entire organism, following administration of polyunsaturated PC claimed that it is associated with an improvement in membrane transport and cellular function.

The magnitude of increase in the phospholipid fraction with cholesterol treatment was far less than that of cholesterol, resulting in a significantly ($p<0.001$) higher cholesterol/phospholipid ratio

Table 5. Total phospholipids and cholesterol/phospholipid ratio in plasma of guinea pigs treated with dietary lecithin and/or cholesterol (means and standard deviations)

Group	Total phospholipids (mg/100 ml)				Cholesterol/Phospholipid			
	Initial	Week 2	Week 5	Week 8	Initial	Week 2	Week 5	Week 8
Control (A)	35.3 ± 5.2	33.8 ± 6.2	38.4 ± 7.2	33.3 ± 5.6	1.31 ± 0.28	1.59 ± 0.33	1.44 ± 0.32	1.89 ± 0.20
Lecithin (B)	34.8 ± 3.0	35.6 ± 7.8	38.2 ± 8.0	38.3 ± 9.5	1.52 ± 0.25	1.39 ± 0.30	1.49 ± 0.28	1.62 ± 0.34
Cholesterol (C)	36.4 ± 4.2	70.8[a] ±30.7	87.4[a] ±42.0	95.0[a] ±47.6	1.59 ± 0.29	2.80[a] ± 0.48	2.64[a] ± 0.23	2.62[a] ± 0.67
Cholesterol + Lecithin (D)	33.5 ± 5.0	91.1[a] ±36.1	103.4[a] ±32.8	90.4[a] ±39.5	1.52 ± 0.09	2.77[a] ± 0.45	2.40[a] ± 0.28	2.99[a] ± 0.42
Statistical comparison (A+B vs C+D)	ns	p<0.001	p<0.001	p<0.001	ns	p<0.001	p<0.001	p<0.001

[a]Significantly different from the initial value, p<0.01

within two weeks and thereafter (Table 5). The ratio of cholesterol/phospholipid did not differ significantly between the groups fed cholesterol with and without the addition of lecithin. This is in contrast to the observations of many investigators (Funatzu, 1966; Morrison, 1958; Adams et al., 1967) who showed a downward change in cholesterol/phospholipid quotient with lecithin supplementation. Although the increased cholesterol/phospholipid ratio has been frequently cited as an indication of atherosclerosis, clinical reports on the validity of using this ratio are contradictory. Nonetheless, cholesterol feeding in guinea pigs enhanced this ratio significantly regardless of the presence of lecithin in the diet.

The absolute amount as well as the proportion of PC in plasma phospholipids is shown in Table 6a; LPC values appear in Table 6b, and the LPC/PC ratio is shown in Table 6c. In the control group, there was no significant change in the amount or proportion of plasma PC or LPC, or in the LPC/PC ratio throughout the experimental period. With lecithin feeding, the absolute amounts of PC, LPC and the LPC/PC ratio showed a slight increase, although they did not attain statistical significance. Halvorson (1976) also reported a trend toward increased PC in plasma of healthy human subjects during lecithin supplementation.

In animals receiving cholesterol, with or without lecithin, the absolute amounts of PC and LPC almost doubled within two weeks ($p<0.01$) and remained high thereafter. At each sampling interval, the responses to the cholesterol-containing diets (C+D) were significantly greater ($p<0.001$) than to the non-cholesterol diets (A+B).

Table 6a. Phosphatidyl choline in plasma of guinea pigs treated with dietary lecithin and/or cholesterol (means and standard deviations)

Group	PC (mg/100 ml)				PC as percent of total phospholipid			
	Initial	Week 2	Week 5	Week 8	Initial	Week 2	Week 5	Week 8
Control (A)	26.6 ± 3.5	26.2 ± 4.0	28.9 ± 4.8	25.3 ± 4.5	75.7 ± 5.1	76.9 ± 4.7	75.6 ± 3.7	76.0 ± 3.7
Lecithin (B)	26.8 ± 2.0	29.3 ± 4.6	29.2 ± 6.3	29.0 ± 8.1	77.0 ± 5.0	76.3 ± 4.1	76.4 ± 3.2	75.3 ± 2.8
Cholesterol (C)	27.9 ± 2.5	52.4[a] ±14.9	63.6[a] ±33.4	67.1[a] ±34.1	77.0 ± 3.8	71.3 ± 5.4	71.7 ± 5.4	70.7[a] ± 3.6
Cholesterol + Lecithin (D)	25.3 ± 3.4	71.4[a] ±28.1	74.6[a] ±26.0	64.5[a] ±27.9	75.7 ± 2.7	73.5 ± 5.6	71.6 ± 3.3	71.4[a] ± 3.7
Statistical comparison (A+B vs C+D)	ns	$p<0.001$	$p<0.001$	$0<0.001$	ns	$p<0.05$	$p<0.025$	$p<0.001$

[a]Significantly different from the initial value, $p<0.01$

Table 6b. Lysophosphatidyl choline in plasma of guinea pigs treated with dietary lecithin and/or cholesterol (means and standard deviations)

Group	LPC (mg/100 ml)				LPC as percent of total phospholipid			
	Initial	Week 2	Week 5	Week 8	Initial	Week 2	Week 5	Week 8
Control (A)	1.46 ±0.29	1.48 ±0.28	1.57 ±0.24	1.37 ±0.25	4.12 ±0.42	4.36 ±0.62	4.20 ±0.82	4.14 ±0.60
Lecithin (B)	1.45 ±0.22	1.71 ±0.47	1.72 ±0.44	1.65 ±0.48	4.16 ±0.57	4.42 ±0.61	4.49 ±0.60	4.27 ±0.30
Cholesterol (C)	1.49 ±0.20	2.41[a] ±0.82	3.10[a] ±1.29	3.09[a] ±1.50	4.10 ±0.43	3.27[a] ±0.51	3.61[a] ±0.40	3.36[ab] ±0.60
Cholesterol + Lecithin (D)	1.38 ±0.29	2.74[a] ±1.16	3.20[a] ±1.14	3.42[a] ±0.87	4.13 ±0.56	2.85[a] ±0.34	3.08[a] ±0.41	4.13 ±1.21
Statistical comparison (A+B vs C+D)	ns	p<0.005	p<0.001	p<0.001	ns	p<0.001	p<0.001	ns

[a]Significantly different from the initial value, p<0.01

[b]C, significantly different from D, p<0.05

Table 6c. Lysophosphatidyl choline/phosphatidyl choline in plasma of guinea pigs treated with dietary lecithin and/or cholesterol (means and standard deviations)

Group	LPC/PC			
	Initial	Week 2	Week 5	Week 8
Control (A)	5.47 ± 0.69	5.66 ± 0.55	5.55 ± 0.98	5.46 ± 0.88
Lecithin (B)	5.40 ± 0.63	5.78 ± 0.68	5.87 ± 0.71	5.66 ± 0.31
Cholesterol (C)	5.34 ± 0.66	4.61 ± 0.78	5.06 ± 0.74[a]	4.77 ± 0.95
Cholesterol + Lecithin (D)	5.46 ± 0.78	3.92 ± 0.69	4.33 ± 0.67	5.78 ± 1.70
Statistical comparison (A+B vs C+D)	ns	$p<0.001$	$p<0.001$	ns

[a] C, significantly different from D, $p<0.05$

Ostwald et al. (1970) showed an almost 5-fold increase in plasma LPC when they treated guinea pigs with 1% cholesterol: the increase of LPC was at the expense of PC in the plasma. It appears that the more pronounced increase in LPC fraction in the experiment of Ostwald et al., as compared with the present study, may be due to the use of a much higher dose of cholesterol in the diet.

In contrast to the increase in absolute amounts of PC and LPC with cholesterol feeding, both fractions fell relative to the total phospholipids. Thus, other phospholipids must have accounted for the high concentrations of total phospholipids. A relative as well as absolute decrease in the LPC fraction has been observed in human atherosclerosis (Gillett and Besterman, 1975) and in spontaneously-induced atherosclerosis in rhesus monkeys (Mohan and Chakravarty, 1975). Thus, the lowered relative proportion of LPC in guinea pigs despite the atherogenic cholesterol treatment in the present study is in contrast to many reports dealing with nutritionally-induced atherosclerosis (Portman et al., 1970). The small amount of cholesterol used in the diet must be responsible for the results obtained in the present study. With cholesterol feeding, the increase in plasma concentration of LPC was less than that of PC, thus producing a significantly lower LPC:PC ratio ($p<0.001$) at the second and fifth week. It was interesting to note that LPC as percent of total phospholipids as well as the LPC/PC ratio in the group treated with cholesterol + lecithin became comparable to that of the groups without cholesterol treatment at 8 weeks, despite the previous reduction of both parameters. This corresponds to the

result obtained from the lecithin group in which % LPC as well as the LPC/PC ratio tended to be slightly higher than the control group. The significance of this observation will be discussed in light of LCAT behavior.

LCAT activity

The time course changes in two measures of LCAT activity are presented in Table 7. The fractional esterification rate is the percent of plasma cholesterol esterified per hour: the net esterification rate is the micromoles of cholesterol esterified per liter per hour. The fractional rate of esterification in control animals remained in a narrow range throughout the experimental period. With the addition of lecithin to the control diet, a significant increase in activity over the initial value was seen after 2 weeks and 5 weeks on the diet. The present data confirm the finding of Takeuchi and Yamamura (1973), in which an increased LCAT activity was observed when the rat received 1% lecithin for one week. In their study, the in vitro addition of lecithin to the incubation medium also increased cholesterol esterification. The postulated mode of action is that polyunsaturated lecithin increases the percentage of linoleic acid in the lipid fractions, thus facilitating the transesterification (Skorepa et al., 1974; Svanberg et al., 1974).

The response of the net esterification rate to lecithin treatment was similar to that of the fractional esterification rate ($R = 0.630$). The increase in free as well as total cholesterol in the plasma of the control group at 8 weeks (Table 4) resulted in a

Table 7. Plasma LCAT acitivity in guinea pigs treated with dietary lecithin and/or cholesterol (means and standard deviations)

Group	Fractional esterification rate (%)				Net esterification rate (micromoles esterified/ l /hr)			
	Initial	Week 2	Week 5	Week 8	Initial	Week 2	Week 5	Week 8
Control (A)	5.64 ±2.42	5.34 ±1.48	5.56 ±1.82	5.45 ±1.06	14.66 ± 4.29	17.00 ± 5.28	16.68 ± 6.95	19.78[a] ± 3.26
Lecithin (B)	4.84 ±1.19	6.37[a] ±2.11	5.78[a] ±1.72	5.48 ±1.13	14.10 ± 3.05	19.41 ± 7.45	18.18 ± 5.64	18.72 ± 4.99
Cholesterol (C)	4.70 ±1.00	1.63[b] ±0.88	1.01[b] ±0.40	1.24[b] ±0.40	14.90 ± 3.90	21.52 ± 6.40	16.91[c] ± 5.51	22.83[a] ± 6.91
Cholesterol + Lecithin (D)	5.26 ±1.01	1.45[b] ±0.52	1.34[b] ±0.69	1.39[b] ±0.58	14.15 ± 2.80	27.73[a] ± 9.55	25.42[a] ± 8.61	27.26[a] ± 3.90
Statistical comparison (A+B vs C+D)	ns	p<0.001	p<0.001	p<0.001	ns	p<0.05	ns	p<0.025

[a]Significantly different from the initial value, p<0.05

[b]Significantly different from the initial value, p<0.01

[c]C, significantly different from D, p<0.05

significantly enhanced net esterification rate over the initial level.

Feeding the atherogenic diet significantly lowered the fractional rate of esterification to one third the initial level as early as 2 weeks ($p<0.01$). Conversely, the net esterification rate, although variable, increased significantly by the 8th week ($p<0.05$). Thus far, the studies on the effect of cholesterol feeding on LCAT activity yield conflicting results. Shapiro et al. (1968) reported that LCAT activity, expressed as a net esterification rate, in the serum of cholesterol-fed rabbits, rats and chickens was considerably inhibited. The merit of this study is limited because of the long incubation periods used in the assay. With extended incubation, the observed activity tends to be influenced by substrate levels and possible product inhibition (Norum, 1974). No change in LCAT activity in the rat (Takeuchi and Yamamura, 1973), and elevated LCAT activity in the squirrel monkey (Portman et al., 1970) have also been reported to result from cholesterol feeding. The response to cholesterol feeding seems to vary from species to species.

The addition of 1% lecithin to the atherogenic diet did not prevent the drastic reduction in the fractional esterification rate at 2 weeks and thereafter. The fractional rate of esterification was slightly higher at 5 weeks and 8 weeks than in cholesterol group, since the latter showed a further decrease in activity with time. The net esterification, on the other hand, was consistently higher in the lecithin-supplemented group than in the cholesterol group after the initiation of the diet. In the cholesterol + lecithin group, the net esterification increased significantly ($p<0.05$) with time.

Compared with the groups receiving no cholesterol (A+B), the cholesterol-fed groups (C+D) displayed a significantly lower fractional esterification rate ($p<0.001$). The increase in the net esterification rate with cholesterol feeding was less dramatic. Thus, one can say that the atherogenic diet produced high levels of circulating cholesterol, especially free cholesterol, and that the precentage of cholesterol that could be esterified by LCAT action declined. On the other hand, there was a responsive increase in the absolute amounts of cholesterol esterified by LCAT and this increase was enhanced by simultaneous lecithin feeding.

In the human, a difference in LCAT activity between healthy subjects and those with coronary heart disease was revealed when the fractional rates of esterification were compared, but not the mean net esterification rate (Soloff et al., 1973). The percent esterification was lower in the coronary group. When the rate of cholesterol esterification was plotted against each subject's free cholesterol concentration, the difference between the two groups became highly significant. In both cases, there was a positive relationship between percent cholesterol esterification and serum free cholesterol concentration. The lowered efficiency of cholesterol esterification in the coronary group as compared to that of the healthy individual was attributed to enzyme deficiency as well as to a change in substrate reactivity.

It would be possible to differentiate enzyme activity from the effect of substrate concentration by use of the Glomset (1969) procedure. In this assay, heated plasma in excess amounts serves as a

common substrate, thus rendering possible a comparison of enzyme activities. However, data obtained by this method are difficult to compare, since the sources of substrate differ within and between different laboratories. Moreover, heat inactivation partially destroys substrate response in a non-uniform manner (Soloff et al., 1973). In the method of Stokke and Norum (1971), which was adopted in the present study, esterification is dependent on the amount of enzyme and also on the concentration and quality of the available substrates. It is believed to closely represent the in vivo condition. However, the differentiation of enzyme activity from substrate availability is not possible.

If the role of LCAT as part of a removal mechanism for excess cholesterol (Glomset, 1968) is assumed, the increased cholesterol in the plasma with cholesterol feeding should be accompanied by increased LCAT activity under normal conditions. As was shown by the decreased fractional rate of esterification of cholesterol-fed animals, the relative enzyme activity in comparison to the amount of free cholesterol in the plasma was far lower than in the control animals. The liver is believed to be the site of the production of LCAT (Glomset, 1968). Impaired hepatocellular function as a result of cholesterol feeding in guinea pigs (Drevon and Hovig, 1977) may have resulted in the production of inadequate enzyme as well as plasma transport lipoprotein. It appears that a significant increase in net esterification rate over initial level as a result of addition of lecithin to the cholesterol diet was not enough in magnitude to accommodate the great increase in plasma cholesterol.

When all of the data were pooled (Appendix Table i), the fractional esterification rate was positively correlated with the percent of esterified cholesterol in plasma and negatively correlated with the percent of free cholesterol ($p<0.01$). In other words, with a rise in the enzyme activity, there is a concomitant rise in the proportion of product, i.e., cholesterol ester. The net esterification rate, on the other hand, was positively correlated with the concentrations of both free cholesterol and ester cholesterol ($p<0.01$). This could be interpreted to mean that a rise in free cholesterol (substrate) induced a rise in LCAT activity, producing a rise in cholesterol ester (product). Both modes of expressing LCAT activity have their uses. The net esterification rate indicates the absolute amounts of free cholesterol that can be esterified per hour. On the other hand, the fractional esterification rate points up the capability of the enzyme to cope with excessive levels of substrate. While most investigators are in favor of using the net esterification rate, Lacko et al. (1974) are of the opinion that the fractional rate of esterification is a more useful parameter for comparative studies than the actual rate of esterification. Since net esterification rate is dependent on the concentration of free cholesterol, large individual variations in free cholesterol concentration generally lead to substantial scatter of the esterification data. In the present study, the lowered fractional esterification rate and the lowered percentage of ester cholesterol in plasma of cholesterol-fed animals would indicate that LCAT activity, although increased, was insufficient to esterify such high levels of cholesterol in plasma.

The assumption has been that LCAT is chiefly responsible for cholesterol esterification in plasma. However, it is difficult to say to what extent the plasma esterified cholesterol is accounted for by the LCAT activity. Miller and Thompson (1973) observed a relatively normal proportion of esterified cholesterol in patients with intestinal malabsorption despite reduced plasma LCAT activity. Clifton-Bligh et al. (1974) also demonstrated an increased proportion of free cholesterol and accelerated cholesterol esterification with Colestipol therapy. These observations seem to be in contradiction to the basic hypothesis that LCAT is mainly responsible for the plasma cholesterol esterification. It should be emphasized that this basic assumption was derived mainly from extremely abnormal conditions, such as familial LCAT deficiency and hepatic disease in which LCAT was either absent or significantly reduced (Gjöne, 1974). Summing up, it appears that there might be factors involved in the regulation of the plasma cholesterol ester fraction other than LCAT activity. For example, esterification of cholesterol in the intestinal mucosa may contribute substantially to the plasma cholesterol ester. Nonetheless, the cholesterol ester fraction in the plasma at any time will represent the net outcome of esterification and transport to and from the tissue.

With regard to phospholipid fractions, it was interesting to note that the increased LCAT activity when lecithin was supplemented to the control diet tended to be accompanied by an increased % LPC as well as LPC/PC ratio (Table 6b and 6c). This is in agreement with the preliminary finding of a parallel change in LCAT and LPC/PC ratio in healthy human subjects with lecithin treatment (Holden, 1976).

By the same token, a significantly lowered fractional rate of esterification in the cholesterol-treated group was associated with a lowered % LPC as well as LPC/PC ratio at weeks 2 and 5. When the data were pooled regardless of time and treatment, a strong positive correlation was shown between the fractional esterification and % LPC as well as LPC/PC ratio (Appendix i). The net esterification rate by LCAT was positively correlated with absolute concentrations of both the substrates, PC and free cholesterol, and the products, LPC and cholesterol ester. Presumably the increased fractional rate of esterification resulted in the increased fraction of LPC in the plasma despite the fast turnover rate of LPC (Portman and Alexander, 1976). However, the relative contribution of tissue LPC resulting from phospholipase action as well as LCAT activity to the plasma LPC concentration was not measured in the present study. Therefore, it appears that increased LPC fraction can be partly, but not entirely, explained by the increased LCAT activity.

According to Dobiásová and Faltová (1974), the increase in the LPC fraction is involved in the rapid transport of plasma cholesterol ester into the liver and other tissues. If this is so, the lowered fractional rate of esterification, by reducing the relative amount of LPC, is partly responsible for the high plasma cholesterol concentration. In view of the large increase of LPC usually associated with nutritionally-induced atherosclerosis in the animals, the relationship between LPC production and cholesterol removal from the plasma is not straightforward. A study of the relationships among the rate of esterification, the metabolism of LPC, and cholesterol turnover should be pursued.

Concentration of tissue cholesterol

An important and recurring question in atherosclerosis studies in man and animal concerns what happens to cholesterol in tissue pools when changes occur in blood cholesterol. In the present study, an attempt was made to learn how dietary cholesterol and lecithin affect the tissue deposition of cholesterol in guinea pigs.

At the time of autopsy, the livers of the cholesterol-treated animals appeared to be discolored, fatty and friable. The data presented in Table 8 show that cholesterol treatment produced an increase in the relative size of spleen and liver, regardless of lecithin supplementation. However, no difference was observed in the relative weights of kidneys, lungs, digestive tract or heart, among the four dietary groups. Ostwald et al. (1974) found the enlarged livers and spleens in cholesterol-treated animals but Yamanaka and Ostwald (1968) also reported an increase in the relative weight of kidneys and lungs of cholesterol-fed guinea pigs. Again, the different levels of cholesterol used in these studies may be responsible for the discrepancies.

The concentration of cholesterol in individual tissues is shown in Table 9. Cholesterol feeding definitely increased the cholesterol deposition in all tissues examined. The concentrations were significantly higher with diets C+D than with diets A+B. Lecithin supplementation to the control diet did not affect the pattern of deposition significantly. On the other hand, the addition of lecithin to the cholesterol diet significantly enhanced the deposition of cholesterol in the liver and total body but produced a lower concentration in spleen.

Table 8. Tissue weights, as percent of body weight, in guinea pigs treated with dietary lecithin and/or cholesterol (means and standard deviations)

Group	Spleen	Kidneys	Lungs	Liver	Digestive tract	Heart
Control (A)	0.11 ±0.00	0.63 ±0.10	0.71 ±0.16	3.80 ±0.52	3.10 ±0.05	0.30 ±0.04
Lecithin (B)	0.10 ±0.00	0.65 ±0.10	0.66 ±0.09	3.80 ±0.59	3.20 ±0.40	0.30 ±0.05
Cholesterol (C)	0.14 ±0.00	0.62 ±0.06	0.84 ±0.17	5.00 ±0.48	3.20 ±0.51	0.30 ±0.02
Cholesterol + Lecithin (D)	0.13 ±0.00	0.61 ±0.06	0.88 ±0.16	5.30 ±0.55	3.20 ±0.51	0.30 ±0.04
Statistical comparison (A+B vs C+D)	$p<0.001$	ns	ns	$p<0.001$	ns	ns

Table 9. Cholesterol concentration in tissues of guinea pigs treated with dietary lecithin and/or cholesterol (means and standard deviations)

Group	Spleen	Kidneys	Lungs	Liver	Digestive tract	Carcass	Total body
Control (A)	3.86[a] ±0.18	3.30[a] ±0.44	2.97[a] ±0.31	2.83[a] ±0.23	1.75[a] ±0.13	165.40[b] ± 11.61	164.46[b] ± 7.97
Lecithin (B)	3.99 ±0.23	3.19 ±0.22	2.96 ±0.34	3.15 ±0.47	1.78 ±0.29	169.95 ± 17.46	176.76 ± 16.07
Cholesterol (C)	6.40[c] ±0.79	3.84 ±0.27	3.73 ±0.61	18.70[c] ±2.98	2.00 ±0.20	187.03 ± 19.37	271.60[d] ± 19.23
Cholesterol + Lecithin (D)	5.85 ±0.47	3.69 ±0.23	3.82 ±0.58	20.87 ±1.67	1.88 ±0.17	196.57 ± 15.62	298.82 ± 14.85
Statistical comparison (A+B vs C+D)	$p<0.001$	$p<0.001$	$p<0.001$	$p<0.001$	$p<0.025$	$p<0.005$	$p<0.001$

[a] mg cholesterol/g wet weight

[b] mg cholesterol/100 g wet weight

[c] C, significantly different from D, $p<0.05$

[d] C, significantly different from D, $p<0.005$

In the experiment of Turley and West (1976), a diet containing 0.24% cholesterol was found to strongly inhibit sterologenesis in the liver and lungs of male guinea pigs. These investigators reported that, compared to liver, the small intestine appeared to make little contribution to total body sterol synthesis, since it showed low sensitivity to the cholesterol negative feedback system. A high cholesterol concentration in the tissue appears to be prerequisite to negative feedback control of cholesterol synthesis. Thus, in the present study, the liver, which responds to cholesterol feeding with diminished synthesis, showed a high concentration of cholesterol, whereas the digestive tract accumulated very little. Another method by which the body copes with a high cholesterol intake is through increased excretion. Green et al. (1976) observed an increased rate of output when the diet contained as little as 0.1% cholesterol. However, the large deposition of cholesterol in tissues (Table 9) attests to the fact that neither decreased synthesis nor increased excretion was sufficient to offset the accumulation of absorbed dietary cholesterol.

Among all the tissues examined, the liver was the chief depot of cholesterol. Most of the increase in body cholesterol of the cholesterol-fed animals was accounted for by the increase in liver cholesterol, which is in accordance with the finding of Green et al. (1976). According to Drevon and Hovig (1977), most of the increase in liver is in the form of cholesterol ester. These investigators suggest that a relative deficiency of the acid hepatic cholesterol ester hydrolase was responsible for the storage of cholesterol ester

in the livers of cholesterol-fed animals. In fact, the great accumulation of cholesterol ester in guinea pig livers is regarded as a reasonable way of handling the increased amounts of cholesterol in the body (Green et al., 1976).

Spleen was also susceptible to cholesterol deposition with cholesterol feeding, especially in the group receiving cholesterol only. The relative increase in spleen sterol was small compared to the 6- to 7-fold increase in the liver. Furthermore, the increased cholesterol content in this organ would not make any significant contribution to total body cholesterol, due to its relatively small size. Carcass cholesterol was significantly increased in the cholesterol-treated groups. This was contrary to the report of Green et al. (1976) who failed to find an accumulation of carcass cholesterol in guinea pigs treated with 0.1% cholesterol. The decreased fractional rate of plasma cholesterol esterification (Table 7) was directly associated with the high concentration of tissue cholesterol in the animals receiving cholesterol. Possibly the liver tissue had been damaged by cholesterol feeding and this influenced both the amounts and the composition of plasma lipoproteins. Furthermore, the deranged liver would not be able to metabolize cholesterol properly, thus resulting in an increased depositon of cholesterol in tissues.

The total body cholesterol (except heart, aorta and formed blood cells) was approximated (Table 9). Guinea pigs, in general, have 7ml of blood per 100 g body weight. With application of the hematocrit value, plasma roughly constitutes 4 ml per 100 g body weight (Green et al., 1976). The contribution of plasma cholesterol to total body

cholesterol was found to be insignificant. However, high circulating levels of cholesterol appeared to coincide with increased total body cholesterol. The total body cholesterol was increased significantly with cholesterol feeding ($p<0.001$). Furthermore, this increase was more obvious when lecithin was added to the diet ($p<0.005$), due mainly to increased hepatic cholesterol deposition. This observation was unexpected for several reasons. With lecithin, in vitro cholesterol uptake by rat intestine has been reported to be decreased, as a result of expansion of the cholesterol-containing micelles with consequent reduction in cholesterol permeation (Rampone, 1973). Furthermore, Saunders and Sillery (1976) presented evidence strongly suggesting that lecithin inhibits the absorption of bile salts from infused segments of rat ileum; loss of bile salts would enhance the conversion and excretion of cholesterol. If the in vitro condition applies to the in vivo situation, decreased absorption of bile salts should be accompanied by decreased deposition of total body cholesterol. Lastly, the temporary hypercholesteremia following phosphatide infusion in the rabbit was interpreted as a mobilization of tissue cholesterol, which would cause a reduction in tissue cholesterol (Friedman et al., 1957). In the light of the above evidence, the significant increase in total body cholesterol with lecithin supplementation to the cholesterol diet is intriguing. In general, cholesterol metabolism in the whole body is subject to many types of regulation. In addition to absorption, the rate of endogenous synthesis, transport and excretion would be determining factors on the tissue deposition of cholesterol. Thus, a study of total cholesterol turnover during lecithin supplement-

ation would provide information as to the steps at which lecithin elicited the increase in total body cholesterol.

The histological changes in the aorta and heart, as a result of dietary treatment, were examined in randomly chosen samples. Oil-red O staining of the heart sections[1] did not show any definite change in coronary vessels. This may be due to the short period of cholesterol treatment as compared with the 116 day study of Babala and Ginter (1968). The Sudan staining of thoracic aorta revealed that 0.25% cholesterol treatment in guinea pigs for 8 weeks induced plaques especially around the aortic arch. The addition of lecithin to the atherogenic diet resulted in a reduced number of plaques. This is in agreement with the general contention that polyunsaturated lecithin reduces the incidence and severity of aortic atherosclerosis (Howard et al., 1971). The finding of fewer plaques in the aortas of animals treated with lecithin + cholesterol, despite their higher total body cholesterol, points to the importance of local aortic metabolism in the pathogenesis of atherosclerosis. Possibly, as suggested by Patelski et al. (1971), the lecithin supplement to the atherogenic diet resulted in normalization of aortic enzyme activities.

Platelet aggregation

The effects of lecithin and cholesterol supplementation on platelet aggregation were studied in relation to the change in plasma lipid

[1] Examined by Mr. Eric May in Veterinary Diagnostic Laboratory at Oregon State University.

components. Guinea pig platelets are known to be more responsive to the aggregating action of ADP than are those of humans (Constantine, 1966). In the preliminary study, 5×10^{-6}M ADP was shown to induce a progressive decline in the aggregation curve, while 3×10^{-7}M induced mainly reversible, but sometimes biphasic aggregation. Near-threshold concentrations of ADP were shown to induce a reversible aggregation curve, i.e., a decrease in optical density followd by an increase toward the initial level. The typical biphasic aggregation curve shows a decrease in optical density followed by an increase toward the initial level, at which point a plateau occurs, followed by a second phase of decreasing optical density, which becomes irreversible. In the preliminary study, the experimental error of the method was less than 10%.

With a strong concentration of ADP (5×10^{-6}M), the aggregating response was within a narrow range (Table 10). In the cholesterol + lecithin treated group at 8 weeks, there was a significant reduction in the maximal extent of aggregation, compared to the group receiving only cholesterol ($p<0.025$). Otherwise, the extent of aggregation was more or less the same regardless of the duration or type of dietary regimen. None of the correlation coefficients between this parameter and plasma lipid components was significant (Appendix i).

By contrast, the induction of platelet aggregation with the weaker concentration of ADP, 3×10^{-7}M, resulted in a very wide range of responses among the individual guinea pigs (Table 10). The wide distribution of responses at this particular concentration of ADP was due to the existence of three different responses. There were

Table 10. Intensity of ADP-induced platelet aggregation (%) in guinea pigs treated with dietary lecithin and/or cholesterol (means and standard deviations)

Group	5×10^{-6}M ADP				3×10^{-7}M ADP			
	Initial	Week 2	Week 5	Week 8	Initial	Week 2	Week 5	Week 8
Control (A)	79.05 ± 5.45	82.90 ± 7.90	81.5 ± 3.04	81.0 ± 4.53	29.00[a] ±30.31	50.60 ±36.32	33.50 ±33.60	30.50 ±30.62
Lecithin (B)	82.70 ± 3.91	81.80 ± 4.85	80.40 ± 3.35	80.50 ± 4.30	61.80 ±31.0	58.70 ±31.47	32.10 ±31.37	33.60 ±35.48
Cholesterol (C)	82.00 ± 5.54	79.70 ± 3.59	79.30 ± 1.38	83.70[b] ± 5.32	58.60 ±30.36	77.30 ± 3.29	63.70 ±30.84	78.00 ± 5.41
Cholesterol + Lecithin (D)	81.90 ± 4.63	81.40 ± 5.34	81.40 ± 3.60	77.70 ± 4.57	42.40 ±32.38	54.20 ±33.74	46.00 ±39.19	68.90 ±27.92
Statistical comparison (A+B vs C+D)	ns	ns	ns	ns	ns	ns	$p<0.05$	$p<0.001$

[a] A, significantly different from B, $p<0.05$

[b] C, significantly different from D, $p<0.025$

reversible aggregations or irreversible with either a progressive decline or a biphasic pattern. The biphasic response is known to comprise two responses: the primary response to the exogenous aggregating reagent followed by the secondary response caused by the release of endogenous ADP from platelets themselves (Wu et al., 1975). In calculating the extent of aggregation for the biphasic response, the height of the second deflexion was used. Due to the wide variation in the responses, another form of the data is also presented (Table 11). In this table the percent of animals which showed reversible platelet aggregation by the weaker ADP concentration (3×10^{-7}M) is presented.

According to Table 10, the response of the platelets from control guinea pigs to 3×10^{-7}M is almost constant throughout the experimental period. In the lecithin-treated group, the initial response prior to the dietary treatment was significantly intense compared to the control ($p<0.05$). However, the lecithin group tended to respond more like the control animals as time progressed.

Compared to the non-cholesterol groups, the cholesterol-treated groups had a significant increase in sensitivity to ADP. This is apparent from the low number of animals showing reversible platelet aggregation (Table 11). None of the animlas in the cholesterol group showed reversible aggregation at weeks 2 and 8. Correspondingly, the intensity of aggregation was significantly higher compared to the groups without cholesterol treatment. On the other hand, the simultaneous feeding of lecithin with cholesterol generally resulted in a reduced sensitivity of the platelets to ADP aggregation. The aggregation curves of the animals showed some reversible responses, despite

Table 11. Percentage of guinea pigs showing reversible platelet aggregation in response to 3×10^{-7}M ADP upon treatment with dietary lecithin and/or cholesterol.

Group	Initial	Week 2	Week 5	Week 8
Control (A)	75	44	67	75
Lecithin (B)	22	33	75	67
Cholesterol (C)	46	0	22	0
Cholesterol + Lecithin (D)	67	42	50	14

the presence of cholesterol in the diet. Accordingly, the results from the present study indicate that lecithin treatment in guinea pigs tends to decrease the sensitivity of platelets to aggregation induced by 3×10^{-7}M ADP. Sano et al. (1973) demonstrated an eight-fold increase in platelet sensitivity to the aggregating action of ADP in patients during acute myocardial infarction and stroke. In a subsequent experiment, they showed that the minimum effective dose of ADP required to elicit platelet aggregation was much lower after a single dose of cholesterol to rabbits.

The positive correlation between total plasma cholesterol and platelet aggregation by 3×10^{-7}M ADP was significant ($p<0.01$) when all the data were pooled regardless of time or treatment (Appendix i). However, when the data were closely examined, the lessened response in the cholesterol + lecithin group, despite the similar concentrations of plasma cholesterol, does not fully support the idea that cholesterol, per se, is responsible for the heightened sensitivity of platelets to ADP. Wu et al. (1975) are also of the opinion that the concentration of cholesterol itself is not the determining factor for the responses in platelet aggregation. Rather, an interaction of dietary cholesterol with the vessel wall was suspected.

The absolute amount of LPC was positively correlated with platelet aggregation. On the other hand, LPC, as percent of phospholipid, showed a negative correlation ($p<0.01$) with the intensity of platelet aggregation by 3×10^{-7}M ADP (Appendix i). It was interesting to note that % LCAT, but not absolute activity was highly negatively correlated with the platelet aggregation. As was discussed

earlier, the increased % LCAT activity was associated with increased LPC when the data were pooled ($p<0.01$). Therefore it is tempting to accept the proposition of Besterman and Gillett (1971) that LPC in the plasma induces a decreased sensitivity of platelets to the aggregating action of ADP in vitro. However, the fact that a decrease in the proportion but not the absolute amount of LPC was associated with the increased sensitivity of platelets to ADP make this reasoning questionable. The existence of interactions between the plasma components and platelets and the changes in platelets themselves as a consequence of the dietary treatment cannot be overlooked.

The increased sensitivity of the platelets to ADP aggregation in the cholesterol-treated group coincided with the high number of plaques in the aorta. Possibly this was due to the adherence of platelets to the damaged vessel wall, as a result of cholesterol ingestion. Edematous changes in the vessel walls were reported to be accompanied by adhesion of platelets to the endothelial surface after the administration of cholesterol (Sano et al., 1973). Platelets adherent to the endothelial surface may release their intrinsic factors. If the adherent platelets are detached from the endothelial surface and re-enter the blood stream, hypersensitivity of the circulating platelets to the aggregating action of ADP could result. By the same token, the fewer changes in blood vessel walls with the simultaneous inclusion of cholesterol and lecithin may have reduced the sensitivity of platelets to ADP aggregation.

The significance of LPC with respect to the regulation of

platelet function in vivo and the interaction of vessel wall with platelets, as a result of lecithin supplementation are topics worthy of further investigation.

V. SUMMARY AND CONCLUSIONS

This study was undertaken to observe the time course of the effects of dietary lecithin and/or cholesterol on plasma cholesterol esterification and platelet aggregation in guinea pigs. In addition, the pattern of tissue cholesterol deposition in relation to the changes in lipid components of plasma was studied.

Male adult guinea pigs were randomly assigned to four dietary groups: Control (guinea pig chow + 10% coconut oil), Lecithin (control diet + 1% lecithin), Cholesterol (control diet + 0.25% cholesterol), and Cholesterol + Lecithin (control diet + 0.25% cholesterol + 1% lecithin). Blood was drawn at 0, 2, 5, 8 weeks after the initiation of the experimental diets and the animals were sacrificed at the end of 8 weeks. Analyses included: total and ester cholesterol in plasma, total phospholipid, phosphatidyl choline (PC), and lysophosphatidyl choline (LPC) in plasma, and cholesterol in selected tissues and carcass. Activity of the lecithin:cholesterol acyltransferase enzyme (LCAT) was assayed, as was ADP-stimulated platelet aggregation.

With both of the cholesterol-containing diets, the guinea pigs showed significantly reduced food consumption as well as body weight gain as early as 2 weeks ($p<0.05$). The lower weight gain was attributed not only to reduced food consumption but also to decreased food utilization. Lecithin did not exert any influence either on body weight gain or on food consumption.

The concentration of cholesterol in plasma of control animals

increased slightly with the duration of the saturated fat diet. Addition of lecithin to the control diet did not alter either the total plasma cholesterol or the relative proportion of esterified cholesterol. With cholesterol in the diet, plasma cholesterol increased to 3-4 times the control value at 2 weeks and continued to increase gradually thereafter. Unexpectedly, the addition of lecithin to the cholesterol diet failed to suppress the hypercholesteremic effect of cholesterol feeding. A decrease in the ester fraction of plasma cholesterol was seen within two weeks of cholesterol feeding, with or without lecithin, ($p<0.01$) suggesting a limited ability to esterify cholesterol.

The concentration of total plasma phospholipids was not affected by lecithin supplementation to the control diet. On the other hand, plasma phospholipids rose steeply with cholesterol treatment. However, the extent of the rise was less than that of cholesterol, and the ratio of cholesterol/phospholipid increased significantly ($p<0.001$). The concentrations of PC, LPC and the LPC/PC ratio showed a slight increase when lecithin was added to the control diet. With cholesterol feeding, the concentrations of PC and LPC increased but the increase was not as great as that of the total phospholipids. Expressed as percent of phospholipids, both fractions fell. In the group treated with cholesterol + lecithin, LPC as percent of total phospholipids and also the LPC/PC ratio became comparable to that of the groups without cholesterol treatment at 8 weeks, despite the previous reduction of both parameters.

LCAT activity was computed as the fractional esterification rate (percent of plasma cholesterol esterified) and as the net esterification rate (micromoles of cholesterol esterified). Both the fractional and the net esterification rates were enhanced at 2 weeks and 5 weeks of lecithin supplementation to the control diet. Compared with the groups receiving no cholesterol, the cholesterol-fed animals displayed a significantly lower fractional esterification rate ($p<0.001$). Thus, although the net esterification rate increased somewhat with cholesterol feeding, a smaller proportion of the circulating cholesterol was esterified. A responsive increase in the absolute amounts of cholesterol esterified by LCAT occurred when the diet contained lecithin plus cholesterol. The lowered percentage of ester cholesterol in plasma of cholesterol-fed animals would indicate that LCAT activity, although increased in absolute amount, was insufficient to esterify such high levels of cholesterol in plasma. When the data were pooled regardless of time and treatment, there was a strong positive correlation between the fractional rate of esterification and the % LPC as well as LPC/PC ratio. The increased LPC fraction could be partly explained by the increased LCAT activity.

Cholesterol treatment produced an increase in the relative size of spleen and liver, regardless of lecithin supplementation, whereas no differences were observed in the relative weights of kidneys, lungs, digestive tract or heart among the four dietary groups. Cholesterol feeding definitely increased the cholesterol deposition in all tissues examined; the liver was the chief depot of cholesterol, especially in the animals receiving cholesterol and lecithin simultaneously.

The total body cholesterol (excluding heart, aorta and formed blood cells) was increased significantly with cholesterol feeding ($p<0.001$); this increase was even greater when lecithin was added to the diet. On the other hand, histological examination of representative aortas revealed a large number of plaques in the cholesterol-fed animals. Fewer plaques were found in aortas of animals receiving lecithin + cholesterol.

The induction of platelet aggregation with 3×10^{-7}M ADP resulted in a very wide range of values among the individual guinea pigs, due to the existence of three different responses. Except for an intense response prior to the initiation of the diet, platelet aggregation in the lecithin group was similar to that of the control animals. The cholesterol-treated group had a significant increase in sensitivity to ADP; fewer animals in the cholesterol group showed reversible aggregation. The simultaneous feeding of lecithin with cholesterol generally resulted in a reduced sensitivity of platelets to ADP. The intensity of platelet aggregation with 3×10^{-7}M ADP was negatively correlated with the fractional esterification rate and with the LPC fraction of plasma phospholipids ($p<0.01$).

The increased sensitivity of the platelets to ADP aggregation in the cholesterol-treated group coincided with the high number of plaques in the aorta. It is possible that adherence of platelets to the damaged vessel wall caused their sensitization. By the same token, the fewer changes in blood vessel walls, seen when the diet contained both cholesterol and lecithin, may have reduced the sensitivity of platelets to ADP aggregation.

Overall, the beneficial effect of lecithin treatment did not appear to be mediated by a lowering of plasma cholesterol under these experimental conditions. Rather, it was mediated by the increased rate of esterification of cholesterol in the plasma and by the reduction in the sensitivity of platelets to the aggregating action of ADP. Further studies are warranted on the significance of LPC with respect to the regulation of platelet function in vivo and the interaction of the vessel wall with platelets as a result of lecithin supplementation. A study of the relationship among the rate of esterification, the metabolism of LPC, and cholesterol turnover would also be worth pursuing.

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APPENDIX

Appendix i. Correlation coefficients between the independent variables in the plasma

	LCAT %		LCAT (mole)		platelet aggregation (5×10^{-6}M ADP)		platelet aggregation (3×10^{-7}M ADP)	
	n	r	n	r	n	r	n	r
Free cholesterol	135	-.766 **	135	.558**	132	-.144	126	.263**
% free cholesterol	131	-.804 **	131	.523**	122	-.101	117	.355**
Cholesterol ester	135	-.769 **	135	.520**	132	.132	126	.263**
% cholesterol ester	135	.591 **	135	-.507**	132	.117	126	-.187*
Total cholesterol	131	-.704 **	131	.498**	130	-.063	126	.240**
LPC	131	-.646 **	131	.504**	122	-.079	117	.255**
% LPC	131	.511 **	131	-.071	122	-.044	117	-.313**
PC	131	-.682 **	131	.487**	122	-.085	117	.220*
% PC	131	.394 **	131	-.027	122	-.036	117	-.287**
total phospholipid	135	-.590 **	135	.353**	142	-.008	137	.268**
LPC/PC	131	.376 **	131	-.167	122	.072	117	-.032
Platelet Aggregation (5×10^{-6}M ADP)	126	.090	126	-.024				
Platelet Aggregation (3×10^{-7}M ADP)	121	.323 **	121	.057				

* Significant at 5% level

** Significant at 1% level