

## MECHANISM OF CHOLESTEROL-LOWERING EFFECTS OF POLYUNSATURATED FATS

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Atherosclerosis is a disease of the intima of the aorta caused by the formation of lipid-containing lesions which proliferate in the lumen of the artery, eventually leading to obstruction of blood flow. Thrombosis is the end result of such degenerative changes of the intima, and ischemic heart disease usually results from thrombotic obstruction that occurs in the coronary artery lesions. It has been widely recognized that a direct relationship exists between serum lipid levels and atherosclerosis, and that blood cholesterol levels are usually elevated in patients suffering from atherosclerosis. Moreover, according to Smith,<sup>1</sup> precipitation of lipids from specific serum lipoproteins may be a key factor in atherosclerosis in humans. Recognition of such a direct relationship between atherosclerosis and blood cholesterol levels has led to an extensive search for agents which can effectively lower blood cholesterol levels, and consequently many hypolipidemic drugs are being extensively used for the treatment of ischemic heart disease. But in many cases they show serious side effects.

Probably a more physiological approach to this problem would be use of natural dietary factors, and against this background the effect of different dietary fats on blood cholesterol levels assumes considerable significance, because overwhelming evidence has accumulated in the literature establishing the fact that the nature of dietary fats exerts profound influence on the blood cholesterol levels in various species of animals. Thus Kinsell *et al.*<sup>2</sup> had demonstrated that blood cholesterol levels are lowered in humans when plant fats containing very high proportions of unsaturated fatty acids are substituted for animal fats in the diet. It has also been demonstrated that oral or intravenous administration of lecithin isolated from soyabean or sunflower seeds containing very high proportions of linoleic acid prevents hyperlipemia in rats and humans.<sup>3,4</sup>

Clearly, these observations are of far-reaching clinical importance and therefore attempts have been made by many workers to find explanations for the mechanisms through which the blood cholesterol levels are lowered by highly unsaturated fats. Four possibilities have been considered in this respect; they are as follows:

- 1) Reduced absorption of cholesterol;
- 2) Reduced synthesis of cholesterol;
- 3) Increased excretion of cholesterol and its catabolites through bile and feces;
- 4) Redistribution of cholesterol from blood to other tissues.

Abbreviations: GPC, glycerylphosphoryl choline;  $\alpha$ -GP,  $\alpha$ -glycerophosphate; ANS, anilino- $\delta$ -naphthalene sulphonate; HDL, high density lipoprotein.

But the evidence from various laboratories from time to time has never been sufficient to establish any one of them, and, in fact, the results have been rather widely divergent.<sup>5-13</sup>

#### EFFECT OF DIETARY FATS ON THE BILIARY EXCRETION OF CHOLESTEROL

Since bile is the major route of excretion of cholesterol and its catabolites, attempts were made in our laboratory to correlate the excretion of cholesterol through the bile of rats fed polyunsaturated fats with the hypocholesterolemic effects of such fats. The following sets of experiments were designed for this purpose.<sup>14</sup>

##### *Effect of highly unsaturated and less unsaturated fats*

Groups of young rats were given *ad lib.* a normal diet (Hindusthan Lever Pellet) together with 10% safflower oil or coconut oil, for 30 days, after which cannulae were established in the bile ducts of these rats and the rates of bile flow were measured every 30 min. The bile was collected for 3 hr, following which the rats were sacrificed and the blood, liver, and bile were analyzed.

Table I shows that feeding of safflower oil led to striking increase in bile flow and reduced blood cholesterol levels but an increase in the liver cholesterol values, as compared to the feeding of coconut oil. At the same time the concentration of cholesterol per ml of bile as well as the total amounts of cholesterol excreted in 3 hr were markedly higher in the safflower oil-fed rats, while the bile salt values did not show any consistent difference in the bile of the two groups.

In separate experiments, where small amounts of 4-<sup>14</sup>C-cholesterol dissolved in safflower oil or coconut oil were given to such rats 6 hr before cannulation, the radioactivity recovered in the bile salts and in the cholesterol of the bile was consistently higher in the safflower oil-fed rats (Table II).

Analysis of the fatty acid composition of the cholesteryl esters of the liver revealed striking increase in the relative proportions of unsaturated fatty acids in the safflower oil-fed rats, while no such significant differences in the unsaturation of the liver phospho-

TABLE I

EFFECT OF FEEDING SAFFLOWER OIL OR COCONUT OIL FOR 30 DAYS ON THE RATE OF BILE FLOW AND EXCRETION OF CHOLESTEROL THROUGH BILE IN RATS

Oil fed	Serum cholesterol ( $\mu\text{mol/ml}$ )	Liver cholesterol ( $\mu\text{mol/ml}$ )	Bile			
			Volume (ml/hr)	Cholesterol ( $\mu\text{mol/3 hr}$ )	Bile acid ( $\mu\text{mol/3 hr}$ )	Phospholi- pids ( $\mu\text{mol/}$ 3 hr)
Safflower	2.40 $\pm$ 0.059* <sup>1</sup>	10.80 $\pm$ 0.81	0.548 $\pm$ 0.02	0.805 $\pm$ 0.051 (0.595 $\pm$ 0.038)* <sup>2</sup>	15.83 $\pm$ 1.42 (12.31 $\pm$ 1.50)	11.16 $\pm$ 1.18 (7.30 $\pm$ 0.66)
	Coconut	3.48 $\pm$ 0.084	8.67 $\pm$ 0.85	0.481 $\pm$ 0.02	0.502 $\pm$ 0.036 (0.402 $\pm$ 0.022)	15.63 $\pm$ 1.33 (12.86 $\pm$ 1.18)

\*<sup>1</sup> S. E. of the mean.

\*<sup>2</sup> Figures in parentheses, values expressed per ml.

TABLE II

EXCRETION OF RADIOACTIVE CHOLESTEROL AND BILE SALTS IN THE BILE OF RATS GIVEN 4-<sup>14</sup>C-CHOLESTEROL DISSOLVED IN OILBile cannulation was done 6 hr after <sup>14</sup>C-cholesterol administration and collection of bile was for 3 hr.

<i>Oil fed</i>	<i>Cholesterol (cpm)</i>	<i>Bile salt (cpm)</i>
Safflower	855 ± 58* <sup>1</sup> (664 ± 42)* <sup>2</sup>	2,191 ± 161 (1,388 ± 88)
Coconut	622 ± 46 (471 ± 41)	1,661 ± 85 (1,164 ± 83)

\*<sup>1</sup> S. E. of the mean.\*<sup>2</sup> Figures in the parentheses, values expressed per ml.

TABLE III

FATTY ACID COMPOSITION OF LIPIDS IN LIVER AND BILE OF RATS FED SAFFLOWER AND COCONUT OIL FOR 30 DAYS

<i>Fatty acid</i>	<i>Liver cholesteryl ester</i>		<i>Liver phospholipids</i>		<i>Bile phospholipids</i>	
	<i>Safflower oil</i>	<i>Coconut oil</i>	<i>Safflower oil</i>	<i>Coconut oil</i>	<i>Safflower oil</i>	<i>Coconut oil</i>
C <sub>12</sub> :0	2.54	2.30	—	—	—	—
C <sub>14</sub> :0	4.30	4.29	—	—	—	—
C <sub>16</sub> :0	26.55	31.95	16.26	21.47	40.99	48.04
C <sub>18</sub> :0	11.04	12.36	39.92	42.71	11.67	13.30
C <sub>18</sub> :1	16.58	34.37	8.40	10.35	5.77	13.30
C <sub>18</sub> :2	38.88	15.51	21.62	13.98	41.55	25.01
C <sub>20</sub> :4	—	—	13.77	11.47	—	—

lipids were noticed in the two groups. In sharp contrast, the fatty acids of the biliary phospholipids revealed a perceptible difference in that, while linoleic acid was the major fatty acid in the safflower oil-fed rats, the coconut oil-fed animals showed palmitic acid as the major fatty acid (Table III).

#### *Effect of highly unsaturated and less unsaturated lecithin*

In another set of experiments, groups of young rats were given the pellet diet *ad lib.* and either no phospholipid or 500 mg of soya phospholipid (EPL) or egg phospholipid per rat per day for 7 days. At the end of 7 days, the bile was similarly collected for 3 hr, after which the animals were sacrificed and the blood, liver, and bile were analyzed. Here, the blood cholesterol level was lower in the control (receiving no phospholipid) and soya phospholipid-fed rats as compared to the egg phospholipid-fed animals, while the liver cholesterol values were comparable in all the groups. But it was most significant that feeding of either type of phospholipid markedly stimulated the rate of bile flow, which was highest in the EPL-fed animals. The total amounts of cholesterol excreted in 3 hr were also appreciably higher in the rats given EPL, while the concentration of cholesterol per ml of bile did not show much difference in the rats receiving EPL and egg phospholipids.

Here also, the fatty acids of the cholesteryl esters and triglycerides of the liver were

more unsaturated in the rats given EPL, while the fatty acid composition of the liver phospholipids showed little difference in the two groups. In striking contrast, the fatty acids of the biliary phospholipids revealed perceptible changes in that there were markedly higher proportions of linoleic acid in the bile of rats given EPL, as compared to the other two groups.

#### ABSORPTION OF DIETARY LECITHIN

The most important aspects of these experiments were that feeding of unsaturated lipids led to considerable increase in the rate of bile flow and in the unsaturation of biliary phospholipids also in increased excretion of cholesterol through the bile. These considerations led us to investigate the mode of absorption of dietary phospholipids and the possible contributions of dietary fatty acids and phospholipids to the synthesis of biliary phospholipids.

Although the mechanism of intestinal absorption of triglycerides has attracted extensive attention of many investigators for a long period of time, there has been relatively little work on absorption of phospholipids. While earlier workers had demonstrated that dietary phospholipids undergo extensive hydrolysis in the lumen of the small intestine, more recent work of Scow *et al.*<sup>15</sup> and of Nilsson<sup>16</sup> with lymph-cannulated rats has shown that about 80% of the radioactivity of the orally administered fatty acid-labelled lecithin can be accounted for in the lymph triglycerides, the rest of the radioactivity being recovered in the lymph lecithin. On the other hand, very recently Parthasarathy *et al.*<sup>17</sup> have demonstrated that during digestion in the intestine the dietary lecithin is extensively hydrolyzed to free fatty acids, GPC and  $\alpha$ -GP, after which the free fatty acids and  $\alpha$ -GP are reassembled into triglycerides *via* the Kennedy pathway.

The physicochemical state of the lipids during digestion of fats in the intestine has also attracted some attention of earlier workers. Lipids as such are insoluble in water; in order that enzymes and cells in general can deal with them effectively, they have to be rendered water-soluble. Hofmann and Borgstrom<sup>18</sup> had put forward the idea that during digestion of a fatty meal the products of lipolysis appear in human intestine in the form of water-clear micellar aggregates, thereby making the dietary lipids water-soluble. But later on,

TABLE IV

LIPID COMPOSITION OF THE FAT AND MICELLAR PHASES IN THE INTESTINE OF RATS FED 20  $\mu$ MOL OF BILE SALTS AND 20  $\mu$ MOL OF CHOLESTEROL IN 0.5 ML GROUNDNUT OIL DISPERSED IN 1 ML WATER WITH EPL\*<sup>1</sup>  
Free fatty acid and cholesterol were present in appreciable amounts in both micellar and fat phases.

EPL fed ( $\mu$ mol)	Micellar phase* <sup>2</sup> ( $\mu$ mol)				
	MG	DG	TG	LPC	PC
30	0.0492	0.073	0.024	0.0234	0.088
40	0.0901	0.057	0.042	0.0392	0.176
Fat phase					
30	0.0194	0.199	0.295	0.010	0.031
40	0.0192	0.211	0.308	0.0135	0.0392

\*<sup>1</sup> Soya lecithin.

\*<sup>2</sup> MG, monoglycerides; DG, diglycerides; TG, triglycerides; LPC, lysophosphatidyl choline; PC, phosphatidyl choline.

Porter and Saunders<sup>19</sup> showed that the methods employed by Hofmann and Borgstrom<sup>20</sup> for the isolation of the aqueous phase of the intestinal contents give rise to several artefacts and at the same time successfully used the technique of ultrafiltration for such purpose.

By isolating the micellar phase of the intestinal contents of rats by the ultrafiltration technique described by Porter and Saunders,<sup>19</sup> it has been demonstrated by us that, following feeding of soya lecithin or egg lecithin in groundnut oil together with bile salts, as compared to the fat phase, the micellar phase contained very high proportions of mono-glycerides but much fewer diglycerides, while almost all of the triglycerides were recovered in the fat phase. The phospholipids, on the other hand, exhibited a rather different pattern in that, both lecithin and lysolecithin were present in much higher proportions in the micellar phase than in the fat phase, with the concentration of lecithin being much higher than that of lysolecithin in the micellar phase (Table IV).

#### INFLUENCE OF DIETARY LECITHIN ON BILIARY LECITHIN

Although a normal diet contains considerable amounts of phospholipids, the possibility of significant contributions of the dietary phospholipids to the synthesis of biliary phospholipids has been largely ignored by earlier workers. Therefore attempts were made in our laboratory to study the pattern of excretion of dietary labelled free fatty acids or phospholipids through the bile of rats. In the experiments where 1-<sup>14</sup>C-linoleic acid was given to bile duct-cannulated rats, nearly 0.1% of the fed radioactivity was recovered in 3 hr in the biliary lecithin, which was in fact the only lipid in the bile to carry the fed radioactivity. It was also observed in these trials that the specific radioactivity of the biliary lecithin was markedly higher than in the liver lecithin, which obviously meant that the exogenous fatty acid was preferentially incorporated into the biliary lecithin. These findings of ours are in general agreement with those of Balint *et al.*,<sup>21</sup> who had claimed that separate pools exist in the liver for the synthesis of biliary and liver lecithin.

In similar experiments where  $\beta$ (1-<sup>14</sup>C-linoleoyl)-lecithin was given, the bulk of the radioactivity present in the biliary lipids was recovered in the lecithin fraction, with virtually all of the radioactivity of the biliary lecithin being retained at the  $\beta$ -position (Table V). Such a situation can arise if the exogenous lecithin molecule escapes the attack of phospholipase A<sub>2</sub> in the intestine and is consequently absorbed intact. Alternatively, following attack of phospholipase A<sub>2</sub> the lysolecithin formed may be reacylated with the same fatty acid which is split off by the enzyme. But it has already been shown that large amounts of lecithin appear in the micellar state in the intestine of rats given lecithin, which would

TABLE V  
DISTRIBUTION OF <sup>14</sup>C-FATTY ACID IN THE  $\alpha$ - AND  $\beta$ -POSITIONS OF BILIARY LECITHIN IN RATS GIVEN 20.0  $\mu$ MOL OF  $\beta$ -(1-<sup>14</sup>C-LINOLEOYL) LECITHIN\*

Time after feeding (min)	$\beta$ -Fatty acid (cpm)	$\alpha$ -Fatty acid (cpm)
90	1,200	170
150	1,930	150
210	1,960	130
270	1,500	200
330	1,557	150

\* Sp. activity = 80,000 cpm/ $\mu$ mol.

TABLE VI

$^{14}\text{C}$ - AND  $^{32}\text{P}$ -RADIOACTIVITIES IN THE BILIARY LECITHIN IN RATS GIVEN  $\beta$ -(1- $^{14}\text{C}$ -LINOLEOYL) LECITHIN- $^{32}\text{P}$  WITH A  $^{14}\text{C}/^{32}\text{P}$  RATIO OF 1

Bile collection started 30 min after administration of the doubly-labelled lecithin. Recovery in 300 min:  $^{14}\text{C}$ =0.3% of dose,  $^{32}\text{P}$ =0.1% of dose.

Time (min)	$^{14}\text{C}$ (cpm/sample)	$^{32}\text{P}$ (cpm/sample)	$^{14}\text{C}/^{32}\text{P}$
60	863	131	6.58
120	1,056	327	3.35
180	1,638	514	3.18
240	1,139	608	1.87
300	1,264	756	1.67

strongly suggest that some proportions of exogenous lecithin are probably absorbed intact. Such a possibility was supported by the results of our experiments on biliary excretion of exogenous doubly-labelled lecithin. In these experiments, where rats were given  $\beta$ (1- $^{14}\text{C}$ -linoleoyl)-lecithin- $^{32}\text{P}$  with a  $^{14}\text{C}/^{32}\text{P}$  ratio of 1, at the initial stages of collection of bile the  $^{14}\text{C}/^{32}\text{P}$  ratio of the biliary lecithin was nearly 7, which declined to a value of nearly 1 at the termination of the experiments at 5 hr (Table VI). These results therefore showed that some proportions of the dietary lecithin are not only absorbed intact, but are transported all the way through the bile in the intact form. The relative loss of  $^{32}\text{P}$  against  $^{14}\text{C}$  in the biliary lecithin, however, was probably due to base-exchange reactions as suggested by Kanoh,<sup>22</sup> Weiss,<sup>23</sup> and others.

#### PHYSICO-CHEMICAL STUDIES OF THE BILE MICELLE

Cholesterol is insoluble in water and it exists in the bile in the form of mixed micelles together with phospholipids and bile salts. Small<sup>24</sup> had concluded from X-ray and NMR studies that phospholipid: bile salt mixed micelles are disc-shaped bimolecular leaflets of lecithin molecules which form the core of the micelle. The ends of the discs are covered by the hydrophilic choline [ $\text{N}^+(\text{CH}_3)_3$ ] groups, while the sides consist of hydrocarbon alkyl chains. The steroid nucleus of the bile salt molecule is hydrophobically associated with the alkyl chain of the lecithin molecule while its hydrophilic parts orient towards the aqueous phase. It has been suggested that, being highly hydrophobic in nature, cholesterol associates itself with the hydrocarbon residues of the phospholipids of the micelles.

The marked increase in the unsaturation of the fatty acids of the biliary lecithin and the increased concentrations of cholesterol of the bile of rats given unsaturated fats led us to compare the mode of packing of the lipids in the bile micelles of rats receiving unsaturated and saturated fats, and these investigations were further extended to an examination of the artificial model micelles made of cholesterol, sodium deoxycholate and lecithin of different fatty acid composition.

The NMR spectra of natural bile fluid of rats closely resembled those of the model micelles prepared with bile salt, cholesterol, and lecithin. The bile salt molecules in the micelles appeared rigidly packed, while the phospholipid molecules were in a fluid state which was evident from the overwhelming contributions of the NMR signals of phospholipids. The natural bile micelles seem to be quite stable: Heating, sonication, or treatment

with protease or phospholipase A<sub>2</sub> failed to effect any perceptible changes in the gross appearance of the spectra. Addition of ANS, however, perturbed the micelles and led to the appearance of steroid signals and an upfield shift of the choline methyl signals. The only significant difference observed in the NMR spectra of the bile from rats fed saturated and unsaturated fats was the increase in the line-width of the choline resonance in the case of the latter samples. Similar broadening of the choline resonance was also observed in model phospholipid: bile salt mixed micelles following incorporation of increasing amounts of cholesterol. These NMR studies have also shown that mixed micelles containing unsaturated phospholipids exhibit greater lipid mobility than those containing dipalmitoyl lecithin. Further, incorporation of cholesterol led to greater restriction of the fluidity of the phospholipids in the unsaturated phospholipid micelles as compared to the micelles containing dipalmitoyl lecithin, thereby suggesting more effective packing of cholesterol in the micelles containing unsaturated phospholipids.

Parallel studies using fluorescent probes like ANS, pyrene, and dansyl cadaverine have lent further support to our conclusions that the matrix of mixed micelles made of polyunsaturated lecithin is more disordered than those prepared from less unsaturated lecithin. Similar conclusions were drawn from a comparative study of the bile of chickens, goats, and sheep. Analysis of the fatty acid composition of the lecithin of these bile samples revealed that, compared to sheep and goat bile, the lecithin of chicken bile is more unsaturated and contains more cholesterol per unit volume of bile. At the same time, it was also found that the matrix of chicken bile micelles is considerably more fluid than that of sheep and goat bile micelles. All these observations are in conformity with our views that cholesterol is more effectively packed in the matrix of the micelles of polyunsaturated lecithin.

#### PHYSIOLOGICAL IMPLICATIONS

The physiological implications of these findings and the overall picture that has emerged regarding the cholesterol-lowering effects of polyunsaturated lipids are schematically presented in Fig. 1 and are briefly discussed below.

It is clear from our work that intake of polyunsaturated lipids in the form of safflower oil or soya phospholipid leads to a striking increase in the rate of bile flow and in the unsaturation of the biliary phospholipids; these in turn solubilize more cholesterol, as a consequence of which higher amounts of cholesterol are excreted through the bile. On the other hand, earlier workers have shown that considerable amounts of cholesterol are excreted into the intestine, where they undergo extensive bacterial modification and are eventually excreted through the feces.<sup>25</sup>

Let us now consider the effects on tissue cholesterol. Cholesterol is transported in the blood by the circulating lipoproteins, and numerous studies have shown that the plasma lipoproteins, especially the HDL, play an important role in the efflux of cholesterol from arterial smooth muscle cells.<sup>26,27</sup> Thus it has been demonstrated that the cholesterol of the arterial smooth muscle cells can freely exchange with the cholesterol of the pre-existing lipoproteins in the fluid environment. In this context it would be pertinent to recall here that in recent years increased fluidity of the lipoprotein matrix has been demonstrated in animals given unsaturated fats,<sup>28</sup> which would obviously mean that, in atherosclerotic animals, feeding of unsaturated fats should lead to a net transfer of cholesterol from the arterial cells to the HDL which have become more fluid due to increase in the unsaturation of its lipids. Indeed, it has been claimed that intravenous infusion of polyunsaturated

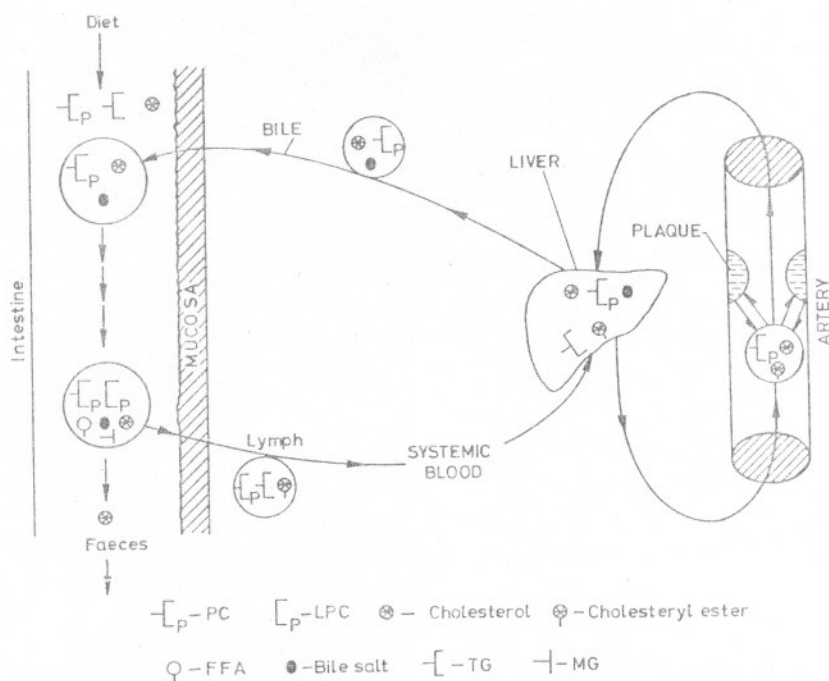


Fig. 1. Dynamics of cholesterol in animal body.

phospholipids in animals can reverse atherosclerosis by removing the lipids deposited in the aorta.<sup>29,30</sup> As we have noted much of the cholesterol thus removed from the tissues is eventually eliminated through the feces *via* bile.

Yet another point which deserves special mention here is that in our work we have failed to notice any significant increase in the conversion of cholesterol to bile salts or in the excretion of bile salts in the rats given polyunsaturated lipids. On the other hand, markedly higher amounts of cholesterol were excreted through the bile of such rats, so that under such conditions the sterol is actually removed as cholesterol and not as its catabolites.

Another equally important point which should be mentioned here regards the solubility of cholesterol in the bile micelles, which is rather critical, because an increase in the ratio of cholesterol: phospholipids or cholesterol: bile salts leads to precipitation of cholesterol causing the formation of gall stones. Therefore it follows that any treatment which enhances the solubility of cholesterol in bile (*e.g.*, an increase in the unsaturation of the biliary phospholipids) should not only prevent gall stone formation but should also dissolve the cholesterol of gall stones. In fact, Dam *et al.*<sup>31</sup> were able to dissolve such gall stones in hamsters by giving margarine rich in linoleic acid.

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