Mode of action of lecithin in suppressing cholesterol absorption

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Abstract In vitro studies were performed to investigate a possible mechanism by which lecithin suppresses intestinal cholesterol absorption. The hypothesis that lecithin acts by retarding the diffusion of micelles across the unstirred water layer (UWL) was tested by measuring cholesterol influxes (J_i) in segments of rat intestine under conditions of maximal and minimal effective thickness of the UWL. J_i was sharply reduced by lecithin under all conditions with no apparent relationship to the thickness of the UWL. The hypothesis was further tested by determining if lecithin had any effect on the molecular weight of the micelles. Gel filtration studies indicated no change in molecular weight (range, 21,000–24,000). Finally, actual measurements of the coefficients of free diffusion (D) of the micelles revealed that lecithin in the concentration that caused a 42–90% reduction in J_i caused only a 22% reduction in D. The basis for the decrease in D is unknown, but it was insufficient to account for the decrease in J_i. Therefore, except for the small decrease in micellar diffusion coefficient as a possible contributing factor, the data offered little support for the hypothesis and we concluded that lecithin suppresses cholesterol absorption by some other or additional mechanism, such as by a direct effect on the cell membrane or by holding the cholesterol in micellar form to reduce its partition coefficient.—**Rampone, A. J., and C. M. Machida.** Mode of action of lecithin in suppressing cholesterol absorption. *J. Lipid Res.* 1981. **22:** 744–752.

Supplementary key words bile salt · diffusion coefficient · flux · gel filtration · micelle · rat intestine · unstirred water layer

Phosphatidylcholine (lecithin) was first shown to suppress cholesterol absorption in rat intestine by Rampone (1) and Rampone and Long (2), an observation that has since been confirmed in several other laboratories in both rat and human studies (3–6). The mechanism by which this suppression occurs has not been determined. Possibilities include *1*) micellar expansion through the formation of macromolecular complexes with cholesterol and bile acids as has been suggested to occur in bile and in artificial systems (7–9) (which would hinder the free diffusion of micelles through the unstirred water layer overlying the villus absorptive surface); *2*) a change in micellar charge or shape in such a way as to similarly hinder micellar diffusion; *3*) a non-specific membrane effect causing a decrease in cell membrane permeability to cholesterol; and *4*) a change in the cholesterol partition coefficient between the cell membrane and the lumen in such a way as to favor retention of cholesterol in the aqueous luminal or micellar phase.

The first of these possibilities seemed the most likely in view of the macromolecular complexes found in bile with which lecithin has been found to be associated (7–10) and because of studies showing that diffusional transfer of cholesterol, and other lipids in micellar form, across the unstirred water layer is the rate-limiting step in the overall absorptive process (11, 12). The present study examines this first hypothesis by determining whether or not the inhibitory effect of lecithin on cholesterol absorption in vitro could be prevented or overcome by reducing the thickness of the unstirred water layer. The idea was based on the following flux equation which describes the influx of cholesterol (J_i) across the cell membrane:

$$J_i = \frac{D}{T}(C_1 - C_2)$$

where D = micellar diffusion coefficient; C_1 = cholesterol concentration in the bulk incubation fluid; C_2 = cholesterol concentration in the unstirred water immediately outside the cell membrane (assuming that the permeability of the cell membrane is very high compared to D so that C_2 also approximates the cholesterol concentration immediately inside the cell membrane (11, 13)); and T = thickness of the unstirred water layer. D is an inverse function of micellar size or molecular weight (M) although the exact relationship is unknown in the case of micelles. Therefore, $J \propto (1/MT)(C_1 - C_2)$ since $D \propto 1/M$.

An increase in M in the presence of lecithin would cause the expected decrease in flux, but a simultaneous decrease in T should prevent or reduce the 'lecithin effect'.

In our experiments we measured J_i in the presence

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and absence of lecithin by incubating rat intestinal segments in the appropriate micellar solutions that were either stirred at a constant rate or left unstirred. Stirring decreases the effective thickness of the unstirred water layer (11) and may expose a greater villus absorptive surface to the micelles.

The results showed that lecithin inhibited cholesterol absorption about equally in stirred and unstirred conditions and, therefore, failed to support the hypothesis. Experiments were then carried out to determine the effect of lecithin on the micellar molecular weights as a criterion of possible micellar enlargement. Finally, studies were carried out to determine the effect of lecithin on the actual coefficient of free diffusion of the micelles in aqueous buffer solution.

MATERIALS AND METHODS

Sources of chemicals

All chemicals were specified as 98+% purity and used as such. Sources were as follows: cholesterol, oleic acid, monooleoyl glycerol, sodium taurocholate, and lecithin (DL-α-dipalmitoyl phosphatidylcholine) were from Sigma Chemical Co., St. Louis, MO; [^{3}H]-cholesterol, [^{14}C]cholesterol, [^{3}H]inulin, and [1-^{14}C]-dipalmitoyl phosphatidylcholine were from Amersham Corp., Arlington Hts., IL; bovine serum albumin, ovalbumin, chymotrypsinogen A, ribonuclease A, blue dextran 2000, and Sephacryl S-200 were from Pharmacia Fine Chemicals, Piscataway, NJ.

Micellar solutions

The lipids, including lecithin when used, were mixed together in benzene which was then evaporated to dryness under a stream of nitrogen. The bile salt was dissolved in a small amount of bicarbonate buffer and added to the dry lipid and the mixture was stirred until a clear micellar solution was obtained. The solution was then brought up to 99 ml with additional buffer. The buffer was Krebs bicarbonate buffer with calcium and magnesium omitted. It was thoroughly equilibrated with 95% O_2, 5% CO_2 gas mixture before use and the final pH was 7.4. Finally, 500 mg of glucose and 1 ml of [^{3}H]inulin (~30 μCi) dissolved in water were added to bring the volume to 100 ml.

Table 1 shows the composition of the micellar solution used in the initial phase of the study to validate the methods. Cholesterol was studied in a range of concentrations, and when its concentration was increased all other components were increased proportionally. An exception was when lecithin was added; to maintain the optical clarity of the solutions (and hence provide assurance that lecithin and the other lipids were in micellar form) it was necessary to increase the bile salt concentration to a minimum of 7.2 mM.

TABLE 1. Composition of micellar solution

Chemical	Concentration
	mM
Sodium taurocholate	4.8
Oleic acid	0.6
Monooleoylglycerol	0.3
Cholesterol	0.15
Glucose	28.0
[^{3}H]Inulin (~30 μCi/100 ml)	

[4-^{14}C]Cholesterol (2 μCi/100 ml) was included as a tracer. [G-^{3}H]Inulin was included in all micellar solutions in approximately 15 times the activity of the ^{14}C-labeled compound to allow for easy discrimination between the two isotopes in the liquid scintillation spectrometer. The inulin served as a marker of the volume of the extracellular adherent fluid, and has been used for this purpose in other laboratories (14, 15).

Procedure

Male Sprague-Dawley rats maintained on standard laboratory chow and weighing at least 200 g were fasted overnight. They were then given an excess of sodium pentobarbital intraperitoneally and, as soon as deep anesthesia was achieved, the small intestine, from the ligament of Treitz caudally, was flushed out with cold physiological saline and removed to a plastic trough filled with cold saline. In the trough it was extended to a length of 100 cm. Twenty-five cm of jejunum and ileum were removed to a beaker containing 200 ml of ice-cold buffer solution through which a continuous stream of 95% O_2, 5% CO_2 was bubbled. They were stored in this way until used. Four segments each (ca. 5 cm in length) of jejunum and ileum were removed from the beaker in random sequence and transferred to a chilled stainless steel plate containing a few drops of buffer. After opening along the mesenteric line, the flat segment was transferred mucosal side out to an oval-shaped platform measuring 20 × 9 mm and 2 mm in height mounted on the end of a 2-cm diameter plastic rod. The apparatus was similar to the one described by Lukie, Westergaard, and Dietschy (14) for rabbit intestine, except that the dimensions were scaled down to accommodate the smaller rat intestine, and the method of holding the tissue on the platform was simplified to obviate the need for screw-clamping the tissue in place with a hollowed-out retaining lid. Instead, the tissue

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was held in place with a tight-fitting plastic cylinder (2 mm thick) having one end hollowed out in the shape of the platform. The intestinal segment overlapped the platform on all sides to provide a leak-proof seal when the outer cylinder was in place. Any excess overlapping tissue was removed to enable the cylinder to be easily placed and to minimize stretching of the tissue.

After mounting and securing the tissue in place, a procedure requiring less than 1 min, the tissue was immersed in cold buffer and stirred for 30 sec to remove excess mucus. This was followed by immersion in unstirred buffer at 37°C for 30 sec for temperature equilibration. It was then quickly transferred to the incubation chamber containing 50 ml of the dual-labeled micellar solution at 37°C, and incubated for time intervals ranging from 4 to 24 min. A continuous flow of 95% O_2, 5% CO_2 was provided through the air space above the incubation fluid to prevent loss of CO_2 and maintain the pH. Initial experiments showed that the pH of the incubation fluid remained unchanged during the course of the experiments. The incubation solution was either unstirred or stirred at a single (standard) rate, controlled by selecting the same setting on the magnetic stirrer in all experiments while holding the tissue the same fixed distance above the stirring bar. Preliminary experiments showed that [^{3}H]inulin required approximately 4 min to equilibrate with the adherent tissue fluid and hence only after 4 min of incubation did it provide a valid measure of the adherent fluid volume. The stirring rate we selected was optimal since in preliminary experiments we showed that, whereas cholesterol influxes were higher in the stirred samples than the unstirred samples, the uptakes could not be further increased by selecting a higher stirring rate.

After incubation, the mounted tissue was quickly immersed in ice-cold buffer for exactly 30 sec to wash away the bulk of the adherent incubation fluid. The wash solution was vigorously stirred with a magnetic stirrer set at the same speed in all experiments. After washing, two circular segments of tissue were punched out using a stainless steel punch having a cross-sectional area of exactly 0.5 cm^2. One ml of Soluene® (Packard Instrument Co., Downers Grove, IL) was added and the vials were immersed in a 37°C water bath until the tissue was completely dissolved. Finally, 10 ml of scintillation cocktail (PPO-POPOP in toluene) was added and the samples were counted in the Packard Model D3320 Liquid Scintillation Spectrometer with red and green channels set to provide maximum discrimination between ^{3}H and ^{14}C, with no spillover of ^{3}H into the ^{14}C channel. A set of [^{3}H]-toluene and [^{14}C]toluene standards was prepared in exactly the same way as the tissue samples, including a non-incubated tissue segment and Soluene® to monitor quenching and to allow counts/min to be converted to absolute activity. These standards were saved and recounted with each new set of tissue samples. Duplicate aliquots of the original micellar solution, similarly prepared, served as the means of converting radioactivity to mass.

The equation for calculating net cellular uptake of the ^{14}C-labeled cholesterol (i.e., uptake over and above the amounts taken up and retained in the extracellular adherent fluid (inulin space)) was as follows:

$$\text{Uptake (nmol/cm}^2\text{)} = 2(a - (bxc/d))/e$$

where a = disintegrations/min (DPM), [^{14}C]cholesterol in the tissue sample; b = DPM, [^{3}H]inulin in the tissue sample; c = DPM, [^{14}C] per μL of incubation fluid; d = DPM, [^{3}H] per μL of incubation fluid; e = specific activity of cholesterol in the incubation fluid, DPM/nmol; and "2" = correction factor needed because uptakes were measured in segments having surface areas of only 0.5 cm^2. All calculations were performed with the aid of the Hewlett-Packard Model 97 programmable calculator. Note that surface area (cm^2) represents serosal surface. No attempt was made to measure mucosal surface area.

Molecular weight determination by gel filtration

The 15-mm diameter column (K15/30, Pharmacia Fine Chemicals, Piscataway, NJ) was packed with Sephacryl S-200 to a total bed volume of 37.5 ml. Blue dextran 2000 (mol wt = 2,000,000) was used to determine the void volume (V_0) and was detected spectrophotometrically at 600 nm. Calibration was performed with the aforementioned proteins which were assayed by the method of Lowry et al. (16) and its absorbance read at 660 nm. Initial studies showed no difference in the elution volume of the test micellar solution when the column was pre-equilibrated either with unlabeled micellar solution or with a simple equimolar bile salt solution. Consequently, data to be reported were derived from columns pre-equilibrated with at least one bed volume of the same molar concentration of bile salt used in the test solutions. Sodium azide (0.02%) was added to retard bacterial growth. All runs were performed at room temperature at a flow rate of 0.25 ml/min as recommended for Sephacryl gel. Mixed micellar solutions were made up as described, omitting [^{3}H]inulin, and were allowed to equilibrate by continuous mixing for at least 18 hr at room temperature. A 0.5-ml volume of this [^{14}C]cholesterol-labeled micellar solution was applied to the top of the gel and eluted with the same solution used for column equilibration. Volumes (0.5 ml) of eluant were collected in preweighed test tubes with the aid of a

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drop counter. After weighing to determine precise volumes, 100-μL aliquots from each tube were counted in 10 ml of Biofluor™ (New England Nuclear, Boston, MA) in the Liquid Scintillation Spectrometer.

The elution volume (V_e) was obtained from the position of the peak maximum of the elution curve. The K_{av} was calculated according to Laurent and Killander (17) as follows: $K_{av} = V_e - V_o/V_t - V_o$, where V_o was determined in triplicate runs of blue dextran 2000 and V_t is the total bed volume.

Determination of the coefficient of free diffusion

Micellar diffusion coefficients were determined by the open-ended capillary method similar to that of Woodford (18) and Wang (19). Eight 10-μL capillary tubes (Dade Diagnostics Inc., Miami, FL) cut to 1.5-cm lengths were filled by capillary action with the radioactive micellar solution and sealed immediately on one end with Seal-ease (Clay Adams Parsippany, NJ). The cholesterol in the micelles was labeled with a trace of [^{3}H]cholesterol (sp act 43 Ci/mmol, not high enough to contribute significantly to the mass of cholesterol). The solutions were optically clear and hence all of the cholesterol was in micellar form. Sodium azide (0.02%) was added to retard bacterial growth.

Each tube was rinsed for exactly 30 sec in Krebs bicarbonate buffer stirred at a controlled rate to remove the small drop of excess micellar solution on the tip of the capillary tube. The contents were then emptied immediately into a scintillation vial by applying air pressure through a tuberculin syringe at the open end of the capillary tube, and counted in Biofluor™. This was repeated four times for each tube as a check for reproducibility of filling and to obtain as precise a measure as possible of the number of radioactive counts contained in the tube at the beginning of the experiment.

The tubes were finally filled once more and incubated in a vertical position for up to 8 days in a large stirred volume (50 ml) of the same micellar solution except for the omission of the radioactive label. After 3–4 days of incubation, duplicate samples were removed daily and the tube contents were emptied and counted as above. The radioactivity remaining in the tube was expressed as a fraction of the original radioactivity, and from this fraction the corresponding value of Dt/l^2 (where D = diffusion coefficient, t = time, and l = length of capillary tube excluding the plug) was obtained from a theoretical plot of the fractional radioactivity remaining versus Dt/l^2 for one dimensional diffusion (19). Thus, from eight capillary tubes we obtained four time points. The experiment was regarded as having proceeded satisfactorily when all four time points agreed.

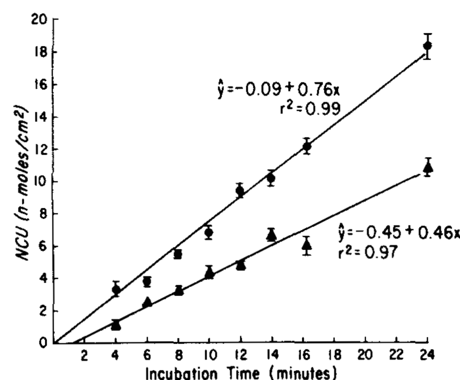

Fig. 1. Net cholesterol uptakes (NCU) after subtracting cholesterol contained in the inulin space in stirred samples at various incubation times in jejunum (●) and ileum (▲). Points are means ± SE in 6–36 duplicate determinations. Concentrations (mM) of micellar components were: cholesterol 0.15, bile salt 4.8, oleic acid 0.6, monooleoylglycerol 0.3.

RESULTS

Using the micellar solution described above (cholesterol concentration = 0.15 mM), net cholesterol uptake was a linear function of the incubation time in both jejunum and ileum (**Fig. 1**); these observations are consistent with other reports (20, 21) and help to validate the methods. Cholesterol influxes, given by the slopes of the regression lines, were significantly higher in jejunum than ileum, possibly reflecting mucosal surface area differences since the fluxes were normalized in terms of serosal, i.e. smooth surface area.

In the following experiments dealing with the effects of lecithin, the incubations were carried out over 6 min and all uptakes were expressed as influxes per cm² of serosal surface area per min (J_i). When lecithin was used, however, the micellar solutions became slightly turbid. To prevent this, the bile salt concentration was increased from 4.8 to 7.2 mM.

Fig. 2 (solid symbols) shows J_i's at a cholesterol concentration of 0.15 mM (bile salt 7.2 mM) in stirred samples as a function of the lecithin concentration. J_i's were suppressed increasingly with increasing lecithin concentration, with complete suppression occurring at 0.5 mM (the slightly negative fluxes were not significantly different from zero). This further validates the method by confirming earlier reports (1–3). Comparing the values on the left at zero lecithin concentration in Fig. 2 (0.81 and 0.55) with the corresponding regression line slopes in Fig. 1 (0.76 and 0.46) shows that the higher taurocholate concentration in the experiments represented in Fig. 2 did not in itself suppress cholesterol influx, contrasting with one report (22) showing a decrease in cholesterol

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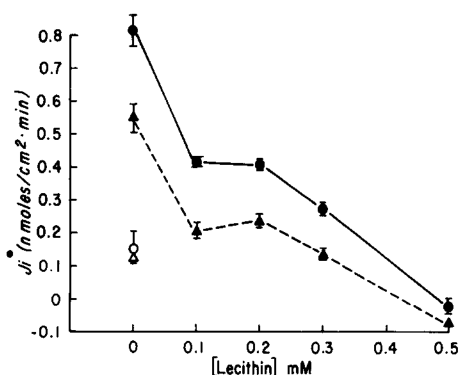

Fig. 2. Cholesterol influx (J_i) in 6-min incubations as a function of lecithin concentration in jejunum (●) and ileum (▲). All samples were stirred at a constant rate except the pair shown by open symbols that were left unstirred. Points are means ± SE in 12–44 duplicate determinations. Concentration of micellar components was the same as in Fig. 1 except bile salt = 7.2 mM and lecithin = 0–0.5 mM.

influx with an increase in the ratio of bile salt (taurodeoxycholate) to cholesterol concentration (see Discussion).

The pair of open symbols in Fig. 2 represents J_i's in jejunum and ileum without any added lecithin but in the absence of stirring. They are presented to show the very low J_i's that were achieved at 0.15 mM cholesterol concentration without stirring, i.e., under conditions of maximum unstirred water layer thickness. The J_i was so low, in fact, that to continue using cholesterol at 0.15 mM would have greatly minimized the chances of showing its further suppression by lecithin in the absence of stirring, a necessary precondition if we were to test the potential reversibility of the lecithin effect by stirring. In the next experiments, therefore, the cholesterol concentration was increased by a factor of three or five (to 0.45 or 0.75 mM), but in order to minimize changes in micellar composition and to keep the cholesterol in micellar form, the concentrations of the other micellar components (oleic acid, monooleoylglycerol, and bile salt) were increased proportionately.

Fig. 3 shows the lecithin effect under these new conditions in stirred (hatched bars) and unstirred (clear bars) samples. All J_i's were increased as expected, but not necessarily in proportion to the increases in cholesterol concentration. Thus, for example, increasing the cholesterol concentration by a factor of 1.67 (0.45–0.75) led to increases in J_i by a factor ranging from near zero (unstirred jejunum in the presence of lecithin, clear bars, column pairs 2 and 4) to 4 (unstirred ileum without lecithin, clear bars, column pairs 5 and 6) and were, therefore, not in accord with prediction based on simple diffusion kinetics. The marked enhancement of J_i by stirring per se is to be noted (hatched versus clear columns). The marked suppression of J_i by lecithin under all conditions is also to be noted, suppression ranging from 25 to 90% in unstirred conditions and from 20 to 70% in stirred conditions. Comparing J_i's at the two cholesterol concentrations, the highest values were always obtained in stirred conditions without lecithin and the lowest in unstirred conditions with lecithin. J_i's obtained in stirred conditions with lecithin always were intermediate between these two extremes, suggesting that the lecithin effect was partially, if not completely, independent of the thickness of the unstirred water layer.

At the top of each pair of columns in Fig. 3 are shown the molecular weights of the differently composed micelles as we determined them by gel filtration. It can be seen that the weights remained within the narrow range of 21,000–24,000 daltons, independent of whether or not lecithin was present. All of the measurements were made in quadruplicate and the repeated runs were in excellent agreement. Adding further to the validity of the measurements was the fact that the weights were the same whether the radioactive tracer was on the cholesterol or lecithin moiety and the fact that the total recoveries of radioactivity from the columns were in excess of 90%, all in a single peak.

The coefficient of free diffusion of micellar choles-

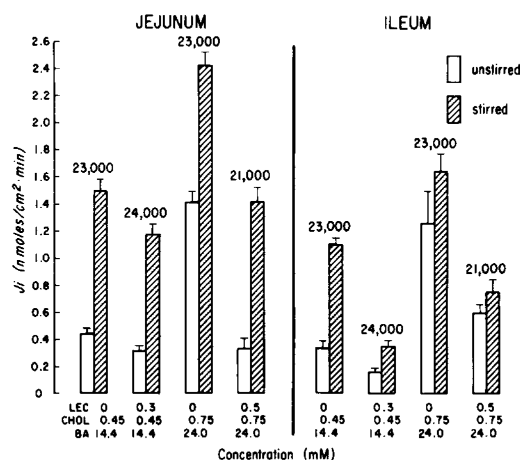

Fig. 3. Effects of lecithin on cholesterol influx (J_i) in 6-min incubations under conditions of minimal (stirred) and maximal (unstirred) effective thickness of the unstirred water layer. Hatched columns, samples stirred at constant rate; clear columns, samples left unstirred. Concentrations of micellar components are as shown. Lec, lecithin; chol, cholesterol; BA, bile acid. For simplicity, oleic acid (concentration 1.8 and 3.0 mM) and monooleoylglycerol (concentration 0.9 and 1.5 mM) have been omitted. Number at top of each pair of columns is estimated molecular weight of the micelles.

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terol in aqueous buffer was determined in the solutions having the highest cholesterol concentration (0.75 mM) in the presence and absence of lecithin as shown in **Table 2.** In the lecithin-free solutions it averaged 1.01×10^{-6} cm²/sec and in the presence of 0.5 mM lecithin it averaged 0.79×10^{-6} cm²/sec, a 22% decrease but considerably less than the up to 90% decrease in the J_i induced by lecithin at the same concentration (Fig. 3).

TABLE 2. Diffusion coefficients (D) of ³H-labeled micellar cholesterol in bicarbonate buffer[a] in the presence and absence of lecithin

Lecithin Concentration	D
mM	*cm²/sec × 10⁶*
0	1.00
0	1.02
	$\bar{X}$ = 1.01
0.5	0.87
0.5	0.71
0.5	0.78
	$\bar{X}$ = 0.79

[a] pH 7.4, 37°C.
Cholesterol concentration was 0.75 mM in all cases and was dispersed into micelles with sodium taurocholate (24 mM), oleic acid (3 mM), and monooleoylglycerol (1.5 mM).

DISCUSSION

These studies have utilized an in vitro measurement system that allows a fairly accurate assessment of some of the factors that determine the unidirectional flux of cholesterol from the intestinal lumen to the interior of the cell. One of the criteria of unidirectionality is linearity of tissue uptake with respect to the incubation time (14, 15, 20, 21). This criterion was satisfied as was shown in Fig. 1; the coefficients of determination (r^2) of the regression lines were 0.99 and 0.97 for jejunum and ileum, respectively. The slightly negative intercepts may be the result of a slight overcorrection for the amount of cholesterol contained in the adherent water layer (inulin space) especially in the shorter incubation times. The basis for the possible overestimate is that the micelles, being larger than inulin, may take longer than inulin to equilibrate in the unstirred water layer. The theoretical considerations associated with the use of reference compounds to correct for adherent fluid have been discussed by Sallee, Wilson, and Dietschy (15).

The cholesterol influxes were nearly twice as high in the jejunum as in the ileum, a difference which we attributed to a difference in mucosal surface area. The fluxes shown in Fig. 1 were normalized in terms of unit serosal surface area; the mucosal surface area was unknown and no attempt was made to measure it. In early experiments we normalized the uptakes also in terms of tissue dry weight and, when this was done, jejuno-ileal differences were much reduced and even difficult to discern in many cases due to the fact that the ileal segments weighed less than the jejunal segments. Thus, in 200 segments the jejunal and ileal dry weights averaged 3.28 ± 0.09 mg/cm² and 2.45 ± 0.09 mg/cm², respectively ($P \ll 0.01$).

Cholesterol influxes at a concentration of 0.15 mM averaged only 0.76 and 0.46 nmol/cm² per min in jejunum and ileum, respectively, even with stirring (Fig. 1). In the absence of stirring, the fluxes were reduced to less than 0.15 nmol/cm² per min in both jejunum and ileum (Fig. 2, ●, ▲), values that we regarded as too low to test our hypothesis adequately. Therefore, we increased the cholesterol concentration to 0.45 and 0.75 mM with proportionate increases in the other micellar components (bile acid, fatty acid, monoacylglycerol). The resulting solutions were optically clear, providing us with assurance that the cholesterol was in micellar form. The J_i's achieved at the higher cholesterol concentrations were increased as expected but not in the proportional fashion that would have been predicted on the basis of simple diffusion kinetics (14, 15, 20, 21). Previous studies have shown that the uptake of cholesterol occurs by a purely passive process (20, 21).

Micelles are highly complex molecular aggregates whose composition, size, shape, and surface charge generally are unknown and probably subject to change depending on conditions such as the relative or absolute concentration of the other lipids and the bile acids in the mixture. In our experiments the cholesterol was radioisotopically labeled and only the flux of the labeled cholesterol was followed. Cholesterol, however, may be polydispersed into multiple aggregate forms of varying size and composition such as simple aggregates with bile salts or complex aggregates with bile salts, fatty acids, monoacylglycerols, lecithin, etc. (23, 24). Its partitioning among the various aggregate forms is likely to vary depending on the composition of the aggregates, and this in turn will affect not only the rate at which the labeled cholesterol diffuses across the unstirred water layer to the cell membrane, but also its partitioning between the aqueous phase or micelles and the cell membrane, and hence its uptake. One or the other of these mechanisms may explain the failure of J_i to increase in linear fashion with increasing cholesterol concentration, as is the case with the passive uptake of simple molecules.

It could be argued that because we increased the concentration of all of the micellar components

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proportionately, keeping their ratios constant, the composition, size, and shape of the micelles would remain constant and only their number per unit volume would increase. The argument may be specious, however, because the micelles were made up of at least four components (bile salt, cholesterol, fatty acid, monoacylglycerol) and we are ignorant about the interactions that might occur in such complex mixtures.

The results in Fig. 3 contrast with those of Westergaard and Dietschy (22). Using simple two-component micelles (cholesterol-bile salt), these investigators found that the J_i for cholesterol in rabbit intestine increased linearly with increasing cholesterol concentration, up to the limit of its solubility, but only if the bile salt concentration (taurodeoxycholate in this case) was held constant to yield a progressively decreasing bile salt:cholesterol concentration ratio. J_i did not increase when the concentrations of both bile salt and cholesterol were increased proportionately, keeping the ratio constant, in sharp contrast to our own constant ratio experiments shown in Fig. 3. The basis for the different results is a matter for speculation but may reside in the complexity of the multicomponent micelles used in our study contrasted with the simple two-component micelles used by Westergaard and Dietschy (22).

Adding lecithin to the mixture adds another level of complexity, particularly in the light of lecithin's amphipathic properties (23). In all cases, the addition of lecithin decreased J_i. The decreases ranged from 20 to 90% (Fig. 3) but the decreases were not consistently related to any single experimental variable that could be identified such as jejunum versus ileum or stirring versus non-stirring. Stirring reduces the effective thickness of the unstirred water layer through which the micelles must diffuse in order to gain access to the cell membrane (11, 12). In stirred conditions, the micelles will gain access to the membrane primarily by bulk or convective transfer and minimally by self diffusion. It was pointed out earlier that the stirring rate we selected was optimal for reducing the thickness of the water layer to its minimum effective value because we achieved maximum influxes at the selected stirring rate. If we assume in the extreme that stirring brought the micelles immediately to the cell membrane by convection and that the unstirred water layer was effectively abolished by stirring, then, since lecithin inhibited J_i regardless of whether or not the solutions were stirred, its effect could not be attributed alone to a slowing of micellar diffusion through the unstirred water layer. We conclude that lecithin must have decreased J_i either by decreasing the permeability of the cell membrane to cholesterol or by preventing the micelles from releasing cholesterol into the aqueous phase or into the cell membrane (in the event of a direct collision with the membrane). The conclusion, however, must be tempered by the fact that such an extreme situation probably does not exist since water that remains trapped between the villi would be only minimally affected by stirring.

In the jejunum, but not the ileum, the presence of lecithin largely, if not completely, abolished the expected concentration-dependent increase in J_i in both stirred and unstirred conditions (compare 2nd and 4th column pairs in Fig. 3). The reason for the observed jejuno-ileal difference is unknown. Possibly lecithin has a selective occlusive effect on the jejunal epithelial cell membrane such as might occur if it were to bind selectively to the jejunal membrane or in some other way prevent cholesterol from gaining access to the jejunal cells.

The experiments in Fig. 3 do not offer any clear support for the idea that lecithin causes micelles to expand or change their shape or mobility in such a way as to reduce their rates of diffusion across the unstirred water layer. Further evidence bearing on this point was sought in two ways: by estimating the molecular weights of the micelles using a gel filtration technique and by actually measuring their coefficients of free diffusion in a few selected incubation solutions.

The molecular weights, shown at the top of the columns in Fig. 3, remained within the narrow range of 21,000–24,000, independent of whether or not lecithin was present, even in the concentration at which it caused the greatest decrease in J_i (0.5 mM). We chose gel filtration because it was least likely to disrupt the micelles or to change their configuration. We took special precautions against this possibility by pre-equilibrating the columns with the appropriate equimolar bile salt solutions and eluting with these same solutions. We also made certain that the flow rate through the columns was as close to 0.25 ml/min as possible during the elution of both the calibrating proteins and the micelles. We conclude that lecithin did not expand the micelles significantly in the concentrations used in our study.

The coefficients of free diffusion, shown in Table 2, were determined only on the micellar solutions having a cholesterol concentration of 0.75 mM with and without lecithin at a concentration of 0.5 mM. Because of initial uncertainties with the method and the long time period required to complete the measurements, we preferred duplicate or triplicate measurements on two samples rather than single measurements on multiple

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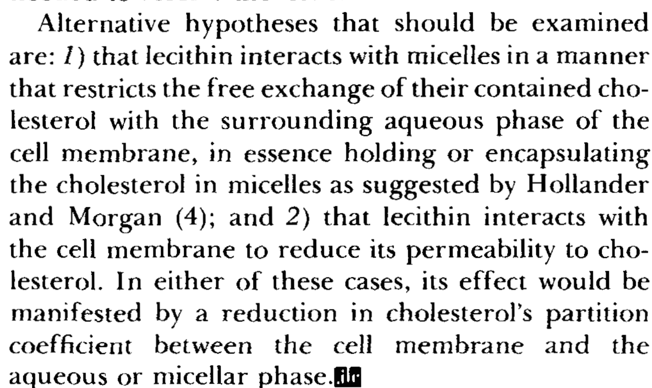
samples. The measurements were consistent and reproducible enough to allow the conclusion that lecithin, in the highest concentration used in the flux experiments, caused a reduction in the coefficient of free diffusion (D) of micellar cholesterol of only approximately 20% (Table 2). In the flux experiments this same lecithin concentration caused a decrease in J_i ranging from 42 to 90% (Fig. 3, column pairs 3, 4 and 7, 8). Thus, the reduction in D in the presence of lecithin was insufficient to account for the reduced cholesterol uptakes, adding further support to the idea that lecithin exerts its major effect via mechanisms other than by retarding the diffusion of micelles across the unstirred water layer.

In the light of the gel filtration studies showing no lecithin-associated molecular weight changes, the basis for the 20% reduction in D must remain speculative. It may be that the gel filtration technique was not sufficiently sensitive to detect changes in molecular weights as small as 20%, or possibly some determinant of D other than molecular weight, such as shape or configuration, was the basis for the reduction in D.

In conclusion, we have found only minimal support for the hypothesis that lecithin inhibits cholesterol absorption by interacting with micelles to cause them to increase in size and slow their rates of diffusion across the unstirred water layer. Micellar weights determined by gel filtration were not significantly increased by lecithin, and the lecithin effect was not influenced significantly by changing the thickness of the unstirred water layer. However, the coefficient of free diffusion of micelles, measured by the open capillary method in one pair of solutions, was decreased by 22% in the presence of lecithin, leaving open the question of whether or not a decrease in D might have been a contributing factor. More experiments, possibly using alternative methods, will be needed to resolve the issue.



Alternative hypotheses that should be examined are: *1*) that lecithin interacts with micelles in a manner that restricts the free exchange of their contained cholesterol with the surrounding aqueous phase of the cell membrane, in essence holding or encapsulating the cholesterol in micelles as suggested by Hollander and Morgan (4); and *2*) that lecithin interacts with the cell membrane to reduce its permeability to cholesterol. In either of these cases, its effect would be manifested by a reduction in cholesterol's partition coefficient between the cell membrane and the aqueous or micellar phase.

This work was supported in part by grant AM21056 from the N.I.A.M.D.

Manuscript received 25 September 1980 and in revised form 26 January 1981.

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