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A mutual inhibitory effect on absorption of sphingomyelin and cholesterol

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Several studies have shown that there is a strong physical interaction between cholesterol and sphingomyelin (SM). The critical factor is thought to be the high degree of saturation in the very long acyl chains of SM. In this study we examined the effects of SM on cholesterol absorption in the rat and compared them with those of phosphatidylcholine (PC). Cholesterol absorption was studied by use of the dual-isotope plasma ratio method. We also studied the effect of sterols on the fecal excretion of undigested SM and its metabolites after a single oral meal of ^{3}H-dihydrosphingosine-labeled SM. When cholesterol was given dissolved in soybean oil, without addition of SM or other phospholipids, absorption was 68 ± 12% in the rat intestine. As a general feature the absorption was less efficient from the cholesterol/phospholipid dispersions. In dispersions with cholesterol and SM, the lowest cholesterol absorption (9 ± 2%) was seen with a cholesterol:SM molar ratio of 1:1. With dispersions of cholesterol and different PC substrates the absorption of cholesterol was lower with saturated PC (16 ± 8%) than with soybean-PC (22 ± 4%) or dioleoyl PC (23 ± 8%). Uptake of SM in the rat intestine was reduced by sterols. For example, percentage recovery of ^{3}H radioactivity in fecal lipids was 38 ± 8% when SM was given with cholesterol and 16 ± 3% without any sterol. One third of the radioactivity in feces was present as ceramide. Sitostanol had the same effect on uptake of SM as cholesterol. This study shows that when rats are fed mixtures of SM and cholesterol the intestinal uptake of both lipids is decreased. By feeding mixtures of SM and sterols the exposure of the colon to ceramide can be increased. (J. Nutr. Biochem. 11:244–249, 2000)

Keywords: sphingomyelin; cholesterol; intestine; absorption; rats

Introduction

The small intestine plays an important role in maintaining cholesterol balance within the body and is responsible for regulating the amount of exogenous dietary and endogenous biliary cholesterol that is taken up by the enterocytes. Of the cholesterol present within the human intestinal lumen over the course of a single day, approximately two thirds are from endogenous sources and one third from the diet. However, excess dietary cholesterol has been shown to raise plasma cholesterol in many individuals.[1] Luminal absorption of cholesterol is incomplete; only approximately 50% of the ingested cholesterol is absorbed.[2] The rate-limiting factors are not well characterized, because the exact mechanism for cholesterol absorption in the small intestine has not been elucidated.

Absorption is strictly dependent on bile salts[3] and is favored by the presence of triglyceride lipolysis products that form mixed micelles with the bile salts, in which the cholesterol can be solubilized.[2] The presence of proteins in the microvillar membrane, with a specific role in sterol absorption, has been postulated[4] but not yet proven. A distinct feature of cholesterol absorption is the ability to distinguish between cholesterol and the plant sterols β-sitosterol and campesterol, which differ from cholesterol

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only in the presence of an extra ethyl or methyl group in the side chain. In several dietary sources of cholesterol and sphingomyelin (SM) the two compounds are associated with each other in some membrane structure (e.g., in meat and the milk fat globule membrane).

Several physical studies have shown that there is a stronger interaction between cholesterol and SM in cell membranes compared with other phospholipids.[5–7] SM and phosphatidylcholine (PC) have the same phosphorylcholine polar head group. However, SM differs in several significant ways from most naturally occurring PCs. The backbone of SM is a sphingoid base (sphingosine is used in this work to designate all sphingoid bases), whereas the backbone of PC is glycerol. This means that the interfacial region of SM is more polar than in PC and allows for stronger intra- and intermolecular hydrogen bonding.[8] The hydrophobic region of SM consists of the relatively short paraffinic residue of sphingosine and an acyl chain that is usually quite long and most often is fully saturated. This means that the hydrophobic region of the SM molecule, in contrast to most naturally occurring PCs, is relatively saturated and asymmetric, with the acyl chain being much longer than the sphingosine chain. However, the asymmetry of the SM molecule is not absolute (e.g., a significant part of milk SM has C16:0 as fatty acid[9]). It is unclear to what extent the asymmetric structure contributes to the interactions of SM with cholesterol.

The aim of the present study was to investigate whether the interaction between SM and cholesterol[10–12] influences the absorption of these lipids in the rat intestinal tract. Cholesterol absorption in rats was studied by use of the dual-isotope plasma ratio method,[13,14] and the effect of sterols on the fecal excretion of undigested SM and its metabolites was studied after a single oral meal of ^{3}H-dihydrosphingosine-labeled SM.

Materials and methods

Chemicals

SM was provided from The Swedish Dairies' Association, which developed a technique for isolating SM using butter milk or whey as raw materials. The technique is protected by a patent.[15] The purity of SM, as analyzed by high performance liquid chromatography (HPLC),[16] was greater than 95%. The composition of SM from bovine milk is complex,[9] with the dominating acyl groups being C16:0 (34%), C23:0 (21%), C22:0 (17%), and C24:0 (14%) and sphingoid bases C18:1 (64%) and C16:1 (23%). Soybean-PC (Epikuron 200; purity >92%) and hydrogenated soybean-PC (Epikuron 200 SH; purity >98%) were provided from Lucas Meyer (Hamburg, F.R.G.). Cholesterol, sitostanol, dioleoyl-PC, soybean-PE, oleic acid, and the ceramide and sphingosine used as standard substances in thin layer chromatography (TLC) analysis were purchased from Sigma Chemical Co. (St. Louis, MO USA). [4-^{14}C]Cholesterol and [1α,2α(n)-^{3}H]cholesterol, dissolved in toluene, were purchased from Amersham (Buckinghamshire, England).

Radiolabeling of SM

^{3}H-dihydrosphingosine-labeled milk-SM (^{3}H-SM) was kindly prepared by Peter Ström (Astra Draco, Lund, Sweden). ^{3}H-SM was prepared by reducing the double bond in the sphingosine part of milk-SM with tritium (RC Tritec AG, Teufen, Switzerland) in PdO/methanol. The specific activity was 48.5 mCi/mg. Purity and identity were determined by ^{1}H and ^{13}C nuclear magnetic resonance (NMR), TLC, and mass spectrometry. Radiochemical purity for SM was greater than 98%, as estimated by TLC on a plate of silica gel developed in $CHCl_3$:CH_3OH:NH_3 (65:35:8, vol/vol/vol). The radioactivity on the plates was located using a RITA92 TLC-scanner (Raytest, Straubenhardt, Germany).

Preparation of labeled cholesterol mixtures

Intravenous preparation. Dispersions with 2.5 μCi ^{3}H-cholesterol per 0.4 mL, contained in Intralipid (20%) from Pharmacia Upjohn (Stockholm, Sweden), were prepared. The required total activity of ^{3}H-cholesterol for each experiment was dried under nitrogen in a glass tube and then redissolved in absolute ethanol (2 μL per μCi of ^{3}H). To this solution was added Intralipid (0.16 mL per μCi of ^{3}H) and the mixture vortexed vigorously for 3 min.

Intragastric preparations. Dispersions with 1 μCi ^{14}C-cholesterol and different amounts of unlabeled cholesterol and milk-SM or PC (soybean-PC, dioleoyl-PC, and hydrogenated soybean-PC, respectively) per 3 mL were prepared. The ^{14}C-cholesterol required for each experiment was transferred to a glass tube and dried under nitrogen. Unlabeled cholesterol was added to the tube and dissolved in 1 mL $CHCl_3$. The solvent was then evaporated under a stream of nitrogen, leaving cholesterol as a thin film on the wall of the glass tube. Dry milk-SM or PC and 0.9% sodium chloride solution was added to the glass tube with the dried cholesterol film and the mixture sonicated for 3 × 1.5 min.

In the first experimental series, dispersions containing 6.5 μmoles milk-SM or PC and three different quantities of cholesterol (2.5, 6.5, and 12.5 μmoles, respectively) per 3 mL were prepared. An additional dispersion contained 6.5 μmoles cholesterol, 6.5 μmoles phosphatidylethanolamine (PE), and 10 μmoles oleic acid per 3 mL Tris buffer (pH 8). (Oleic acid and alkaline pH were used to achieve a lamellar phase with PE.[17])

The second experimental series involved dispersions with a molar ratio cholesterol:SM of 1:1 and containing 12.5 and 25 μmoles of each lipid per 3 mL.

The third experimental series involved dispersion containing 6.5 μmoles cholesterol per 0.5 mL soybean oil, with no addition of SM or other phospholipids. Cholesterol was dissolved in soybean oil by heating the mixture to 37°C for 30 min and then vigorously vortexing for 3 min.

Preparations containing ^{3}H-labeled SM

In a fourth experimental series, three different dispersions containing 3 μCi ^{3}H-SM and 6.5 μmoles unlabeled milk-SM per 3 mL were prepared. To one dispersion was added 6.5 μmoles cholesterol and to another dispersion 6.5 μmoles sitostanol per 3 mL. The third dispersion was made without any addition of sterols. The required total activity of ^{3}H-SM dissolved in ethanol was transferred to glass tubes and dried under nitrogen. Unlabeled milk-SM, sterol (in two dispersions), and 0.9% sodium chloride solution were added to the glass tubes and the dispersions sonicated for 3 × 1.5 min.

Animal experiments

Male white Sprague-Dawley rats, weighing approximately 225 g, were obtained from Möllegaard Breeding Centre (Ejby, Denmark), housed in a temperature-controlled room under a 12-hr light:12-hr dark cycle, and fed a commercial standard pellet diet (Altromin nr 1324, Chr. Petersen A/S, Greve, Denmark) with free access to water.

Table 1 Cholesterol absorption in rats fed mixtures of sphingomyelin (SM) and cholesterol

SM (μmoles; given dose)	Cholesterol (μmoles; given dose)	SM:Cholesterol molar ratio	Cholesterol absorption %; means ± SD	(*n*)
6.5	2.5	2.6:1	17 ± 8	(5)
6.5	6.5	1:1	14 ± 4	(8)
6.5	12.5	0.5:1	25 ± 12	(5)
12.5	12.5	1:1	9 ± 2	(5)
25.0	25.0	1:1	15 ± 10	(5)
	6.5 (dissolved in 0.5 mL soy bean oil)		68 ± 12	(5)

Rats were fed dispersions containing ^{14}C-cholesterol, unlabeled cholesterol, and SM, and then injected with ^{3}H-cholesterol. The absorption of cholesterol was determined 3 days later by analysis of radioactivity in blood serum. For details, see Materials and methods.

Before the experiments rats were fasted for 24 hr, with free access to tap water. The rats were anesthetized lightly with diethyl ether and fed 3 mL of the dispersions (0.5 mL of the dispersion with soybean oil) described above by gastrogavage. All oral dispersions were prepared the afternoon before. On the day of experiments each dispersion was sonicated an additional 1.5 min.

In the first three experimental series, cholesterol absorption was studied by use of the dual-isotope plasma ratio method.[13,14] Immediately after the intragastric feeding with preparations containing ^{14}C-cholesterol, each rat was injected with 400 μL of the Intralipid mixture labeled with ^{3}H-cholesterol, as described above. An incision of approximately 0.5 cm was made on the left side of the breast and the mixture slowly injected into the jugular vein. The incision was then closed with silk sutures. After treatment the rats were fasted another 4 hr with free access to tap water, after which time food was available to them. Three days after dosing, the rats were again lightly anesthetized with diethyl ether and blood from the abdominal aorta was taken into a syringe containing ethylenediamine tetraacetic acid (EDTA) as anticoagulant.

In the fourth experimental series, after feeding the preparations containing ^{3}H-SM by gastrogavage the rats were placed single in cages with wire mesh floors and fasted another 4 hr with free access to tap water, after which time food was available to them. Feces were collected from each rat for two days after feeding from trays placed beneath each cage.

Determination of cholesterol absorption

The blood from each rat was centrifugated and 1 mL aliquots of plasma were taken for liquid scintillation counting and determination of ^{3}H- and ^{14}C-cholesterol levels. The data for ^{3}H and ^{14}C in the plasma samples and in the given intragastric and intravenous doses were used to calculate the cholesterol absorption, by use of the following expression:

Percent cholesterol absorption (14):

$$\frac{\text{Percent of intragastric dose } (^{14}\text{C-cholesterol}) \text{ per mL plasma}}{\text{Percent of intravenous dose } (^{3}\text{H-cholesterol}) \text{ per mL plasma}} \times 100$$

Analysis of lipids in feces

In rats given dispersions with ^{3}H-SM, lipids were extracted from the feces with chloroform:methanol:water, according to the method of Bligh and Dyer.[18] Aliquots of the chloroform phase were taken for liquid scintillation counting. On plates of silica gel (Merck, Si 60-F; 0.25 mm) (Merck Ltd., Dorset, United Kingdom), aliquots of the chloroform phase were taken for TLC analysis. The plates were developed in chloroform:methanol:ammonia (100:15:1.5, v/v/v), which separates SM and its hydrolysis products ceramide, sphingosine, fatty acid, and triglyceride. In this solvent system SM and other phospholipids remain at the start, while triglycerides migrate with the front. Rf values for sphingosine and ceramide were 0.3 and 0.7, respectively. Free fatty acids with an Rf value of 0.1 are included in polar lipids in the presentation of results.

Statistical analysis

The statistical computer software package SYSTAT for Windows (version 5.0) (SPSS Inc., Chicago, IL USA) was used for statistical calculations of data. Values are reported as means ± SD. One-way analysis of variance was used for the calculation of significant differences in the means of multiple groups. Comparisons of data in two groups were made with two-tailed Student's *t*-test for unpaired samples. Differences were considered significant at a *P*-value of less than 0.05.

Results

Cholesterol absorption from dispersions with milk-SM

In experimental series 1, when rats were fed dispersions with 6.5 μmoles milk-SM and three different doses of cholesterol (2.5, 6.5, or 12.5 μmoles), the lowest cholesterol absorption (14 ± 4%) was obtained with a cholesterol:SM molar ratio of 1:1 (*Table 1*), which was significantly lower ($P = 0.025$) than with the higher cholesterol dose (12.5 μmoles). With the cholesterol:SM molar ratio maintained at 1:1, but the dose increased to 12.5 or 25.0 μmoles (experimental series 3), absorption of cholesterol remained at a low level (*Table 1*).

In comparison, when rats were fed 6.5 μmoles cholesterol dissolved in soybean oil, without any addition of SM or other phospholipids (experimental series 3), 68 ± 12% of the cholesterol dose was absorbed, a significantly higher absorption ($P < 0.001$) than with mixtures of cholesterol and SM or PC.

Cholesterol absorption from dispersions with different PC substrates

In continuation of experimental series 1, rats were fed cholesterol dispersions with 6.5 μmoles PC instead of SM. Three different PC substrates were used: soybean-PC (rich in linoeic and linolenic acids), dioleoyl-PC, and hydroge-

Table 2 Cholesterol absorption in rats fed mixtures of phosphatidylcholine (PC) and cholesterol

	Given dose cholesterol (μmoles)		
	2.5 (n = 3)	6.5 (n = 8)	12.5 (n = 3)
	Cholesterol absorption, % (means ± SD)		
PC (6.5 μmoles)			
Hydrogenated PC	24 ± 12	16 ± 8	16 ± 8
Dioleoyl-PC	28 ± 12	23 ± 8	27 ± 15
Soybean-PC	33 ± 3	22 ± 4	36 ± 12

Rats were fed dispersions with 1 μCi ^{14}C-cholesterol and 2.5, 6.5, or 12.5 μmoles unlabeled cholesterol, sonicated with 6.5 μmoles of different PC substrates. After feeding each rat was immediately injected with 3 μCi ^{3}H-cholesterol. The absorption of cholesterol was determined three days later by analysis of radioactivity in blood serum. For details, see Materials and methods.

nated soybean-PC (saturated fatty acids). Although the absorption of cholesterol was significantly lower from the dispersions with PC than with soybean oil, it was influenced by the fatty acid composition of PC. Lowest absorption was obtained with hydrogenated PC (*Table 2*). With a molar ratio of 1:1 for cholesterol and PC the absorption with hydrogenated PC was 16 ± 8%, which was significantly lower (P = 0.031) than with unsaturated PC (dioleoyl-PC, 23 ± 8%; soybean-PC, 22 ± 4%). The same pattern as with SM was obtained, with the cholesterol absorption being lowest at a cholesterol:PC molar ratio of 1:1.

Compared with a mixture of cholesterol and SM in a molar ratio of 1:1, the cholesterol absorption was significantly higher from dispersions with cholesterol and soybean-PC (P = 0.001) or dioleoyl-PC (P = 0.01).

When rats were fed a dispersion with 6.5 μmoles of cholesterol, 6.5 μmoles of soybean-PE, and 10 μmoles oleic acid, at pH 8 (addition of oleic acid and alkaline pH was necessary to get a lamellar phase with PE[17]), the cholesterol absorption was 56 ± 11% (n = 6), which was higher (P < 0.001) than with SM or any of the PC substrates examined.

SM metabolites in rat feces

In experimental series 4, rats were fed dispersions containing 6.5 μmoles milk-SM and 3 μCi ^{3}H-SM. To one dispersion was added 6.5 μmoles cholesterol and to another 6.5 μmoles sitostanol. Data on recovered radioactivity in feces, collected for 2 days, is given in *Table 3*. When rats were fed SM without any addition of sterol, 16 ± 3% of the given radioactive dose was recovered in feces. The recovery of radioactivity increased significant (P < 0.001) to 38 ± 7% in feces from rats fed a mixture of SM and cholesterol in a molar ratio of 1:1. Replacing cholesterol with sitostanol gave almost the same recovery of radioactivity in feces (31 ± 2%) as cholesterol. Approximately one third of the radioactivity in feces was present as ceramide in all three groups (*Table 4*). The total amount of exogenous ceramide in feces thus increased when SM was mixed with a sterol.

Table 3 Recovered radioactivity in feces after feeding rats ^{3}H-sphingomyelin (SM). (%) of given dose)

	Means ± SD	n
SM	16 ± 3	6
SM + cholesterol	38 ± 7	9
SM + sitostanol	31 ± 2	3

Rats were fed 3 μCi ^{3}H-SM with or without addition of 6.5 μmoles sterols. Feces were collected for 2 days after feeding. Recovered radioactivity in feces was analyzed as described in Materials and methods.

Discussion

The main finding of this study was that the absorption of cholesterol from sonicated mixtures with SM or saturated PC is low, as estimated by the dual-isotope method.

When cholesterol was given dissolved in soybean oil, without addition of phospholipids, the absorption was 68 ± 12%. As a general feature the absorption was significantly lower (P < 0.001) from the cholesterol/phospholipid dispersions. In dispersions with the SM:cholesterol molar ratio varying between 0.5 and 2.6, the lowest absorption (9 ± 2%) was seen with a cholesterol:SM ratio of 1:1 (*Table 1*). With dispersions of cholesterol and different PC substrates (soybean-PC, dioleoyl-PC, and hydrogenated soybean-PC), cholesterol absorption was influenced by the fatty acid composition in PC. The same pattern as with SM was obtained, with lowest cholesterol absorption at a cholesterol:PC ratio of 1:1. From dispersions with 6.5 μmoles cholesterol and 6.5 μmoles PC, the absorption was 16 ± 8% with hydrogenated soybean-PC, 22 ± 4% with soybean-PC, and 23 ± 8% with dioleoyl-PC.

Several reports have claimed that natural PCs with unsaturated fatty acids lower the serum cholesterol level.[19,20] A report[20] on feeding rats 5 mg cholesterol and 150 mg linoleic acid-rich PC (Epikuron 200) or 100 mg corn oil showed that there was a greater absorption of cholesterol on the corn oil diet (59.7%) than on the PC diet (41.8%). This is in agreement with the results on soybean oil and soybean-PC in the present study. However, we have also shown that saturated fatty acids bound to certain phospholipids (SM and PC) lower the absorption of cholesterol in rats more than PC with unsaturated fatty acids.

From studies on interactions between cholesterol and SM

Table 4 Sphingomyelin (SM) metabolites in rat feces, percentage of total radioactivity (means ± SD)

	Polar lipids*	Sphingoid bases	Ceramides	Non polar lipids
SM	46 ± 5	5 ± 1	28 ± 1	21 ± 3
SM + cholesterol	46 ± 12	6 ± 4	32 ± 8	16 ± 6
SM + sitostanol	57 ± 9	6 ± 1	25 ± 6	12 ± 5

Rats were given ^{3}H-SM dispersions, with and without addition of cholesterol. Feces were collected for 2 days after feeding. Lipids were extracted from the feces and taken for thin layer chromatography analysis.
*Unesterified fatty acids are included in polar lipids.

in cell membranes, it appeared that the high degree of saturation in the acyl chains of SM and also the 4,5-trans double bond in the sphingosine part, close to the interfacial region, facilitated strong attractive interactions with cholesterol.[10] For PC with hydrocarbon structural similarities with SM (e.g., when at least one of the acyl chains is saturated), strong interactions between PC and cholesterol also occur.[21] In the present study the mixtures of cholesterol and SM or hydrogenated soybean-PC after sonication thus contained vesicles with strong interactions between cholesterol and the phospholipids. This may influence the partitioning of cholesterol to the micellar phase and thus the absorption of cholesterol, parameters that may in turn depend on the partitioning of the phospholipids between the larger aggregates and the micelles, as well as the rate at which the SM and the glycerophospholipids are hydrolyzed. It has been shown that in the equilibrium between liquid crystalline phases (vesicles) and micellar phases in bile, SM seems to partition to bile salt micelles to a small extent[22] and it was suggested that saturated PC probably behaves similarly. The presence of cholesterol might decrease the micellar solubility even more.

Earlier studies indicate that the presence of phospholipids in mixed bile salt micelles retard the absorption of micellarly solubilized cholesterol. Everted sacs and perfused intestinal segments absorbed cholesterol much slower with PC present in mixed micelles than with lyso-PC,[19,23] and in intestinal CaCo2 cells hydrolysis of PC by pancreatic phospholipase A_2 was a rate-limiting factor for the uptake of cholesterol from mixed micelles.[24] The nondigestible diether PC retards cholesterol absorption from bile acid mixtures in bile fistula rats in vivo compared with hydrolyzable PC.[25] In our studies hydrolysis of both PC and SM may thus be rate-limiting factors for cholesterol absorption and the differences in effect may reflect varying rates of hydrolysis of the different phospholipids.

Another finding in our studies was that the presence of cholesterol or sitostanol in a meal containing ^{3}H-SM decreased the absorption of SM. The percentage recovery of radioactivity in feces was significantly higher ($P < 0.001$) when SM was given together with an equimolar amount of cholesterol than given alone (*Table 3*). Sitostanol, a nonabsorbable plant sterol with a structure differing from cholesterol only by an extra ethyl group in the side chain and lack of double bond between carbons 5 and 6, showed the same decreasing effect on the uptake of SM as cholesterol. Approximately one third of the radioactivity in feces was present as ceramide (*Table 4*). The total amount of exogenous ceramide in feces was thus higher when SM was mixed with a sterol. The lack of 4,5-trans double bond in the ^{3}H-labeled SM in this study may to some extent have affected the results.

Dillehay et al.[26] and Schmelz et al.[27] demonstrated that the addition of SM to a normal diet in mice reduced the promotion of colon cancers induced by 1,2-dimethylhydrazine. Although the inhibitory factor has not been established, ceramide may be responsible, because ceramide has been shown to have antiproliferative effects on cell growth and to induce apoptosis.[28] We have recently demonstrated[29] that alkaline sphingomyelinase activity is decreased in human colorectal carcinoma. In experiments on rats we have also shown[30] that the hydrolysis and absorption of SM in the intestine has a relatively low capacity and that oral administration of SM influences the levels of ceramide to which ileal and colonic mucosa are exposed.

If the hypothesis is correct, that ceramide derived from dietary SM may suppress development of colon cancer, it would be of interest to increase the amount of dietary SM and ceramide that reaches colon. Mixing with cholesterol seems to be one way of doing it. In most dietary sources of SM, the two compounds occur associated with each other in a membrane structure, for example, as in the milk fat globule and in meat. Whether this implies that naturally occurring cholesterol and SM also exert a mutual influence on their digestion and absorption remains to be elucidated.

In conclusion, this study has shown that when rats were fed mixtures of SM and cholesterol the intestinal uptake of both lipids were decreased. Hydrogenated PC had nearly the same effect on cholesterol absorption as SM. In addition, PC with unsaturated fatty acids decreased cholesterol absorption compared with absorption from soybean oil. The plant sterol sitostanol reduced the uptake of SM to the same extent as cholesterol.

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