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Influence of Sodium Palmitate in the Solubilization of Cholesterol in an *in vitro* Model of Bile

F. G. Hegardt * and J. L. Vicedo

University of Valencia
College of Alicante
Alicante (Spain)

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The cholesterol solubilizing power of sodium palmitate in aqueous solutions of sodium taurodeoxycholate and lecithin in models *in vitro*, has been explored. From the results, it appears that sodium palmitate increase moderately the cholesterol solubilizing power of the systems and that the relationship between solubilized cholesterol and palmitate concentration is linear, the slope of the straight lines depending upon the concentration of the lecithin present. The regression slope has been quantified and also the equation which relates the increases of cholesterol due to the concentration of the soap, and the concentrations of it and of the lecithin.

It is suggested that the discrepancies between «supersaturation» of cholesterol, according to the models cited in the literature, and the non occurrence of gallstones may be attributable to not taking into account the sodium palmitate concentration in the bile samples under study.

In vitro bile models comprising bile salt, lecithin, cholesterol and water, have been studied by various authors (1, 17, 21, 22), who have tried to quantify the proportion of cholesterol soluble in water at various concentrations of each variable. From these studies and also from that of HEGARDT and DAM (8), the proportions of dissolved cholesterol for each of the six bile salts can be correlated, the quantitative variation of the fatty acid pattern of the lecithin under study being of minor importance (8, 18).

On the other hand, the *in vitro* model of HEGARDT and DAM (8) accounts largely for the occurrence or non occurrence of cholesterol-rich gallstones *in vivo* of several laboratory animals such as chickens, male and female mice, rats, and hamsters fed on very different diets whether «lithogenic» or «curative» (3). However, when an attempt is made to relate the incidence of cholesterol gallstones in man with the proposed model, it fails, since it

* Present Adress: Department of Biochemistry, Faculty of Pharmacy, University of Barcelona, Barcelona - 14 (Spain).

cannot explain the following facts (4, 6): 1) More than 74 % of the patients with peptic ulcers but normal livers and biliary tracts show supersaturation of cholesterol but no gallstones. 2) Fifty per cent of young healthy people studied has no gallstones and apparently show supersaturation of cholesterol. HOLZBACH *et al.* have recently reported similar results (12).

Finally, MUFSON *et al.* (14) agree that the solubility of cholesterol in the system described is not sufficient to explain the cholesterol supersaturation found in the bile of normal men, and suggest that there must be some «forgotten water soluble components that change the status of cholesterol dissolved in bile».

We have assayed the effect of sodium palmitate as an adjuvant to solution of cholesterol in the presence of bile salts and lecithin.

Materials and Methods

Chemicals. Sodium taurodeoxycholate was prepared in our laboratory by the method of NORMAN (19) and purified according to HOFMANN (10). Taurine and deoxycholic acid used as starting materials were obtained respectively from Fluka and Merck. Deoxycholic acid and sodium taurodeoxycholate were tested for purity by thin-layer-chromatography of 200 microgram of application on Silica Gel G (Merck) (moving phase, bencene:ethanol: acetic acid 30:10:2) and found to be pure.

Cholesterol was obtained from Merck. Thit-layer-chromatography on Silica Gel G (moving phase bencene:ethyl acetate: ether:acetic acid 80:10:10:0.2) did not reveal the presence of impurities.

Lecithin was purified chromatographycally from fresh egg yolks (9) by a modification of a previously described method (20). The final product was tested by thin-layer-chromatography on Silica Gel G (moving phase chloroform:methanol:water 100:40:6) and was found to be pure.

Table I. *Fatty acid pattern of the chromatographycally purified egg yolk lecithin used in the experiments.*

Fatty acids	Percentage
C_{14} : 0	0.36
C_{16} : 0	31.91
C_{16} : 1 ω 7	0.81
C_{18} : 0	13.03
C_{18} : 1 ω 9	32.84
C_{18} : 2 ω 6	16.43
C_{20} : 4 ω 6	1.67
C_{22} : 6 ω 6	2.96

The fatty acid pattern of this egg yolk lecithin is shown in Table I.

Sodium palmitate was prepared immediately before use, dissolving the palmitic acid in an appropiate neutralizing NaOH solution (phenolftalein) and maintaining it dissolved in an ethanol water mixture.

Preparation of ampoules. Chloroform solutions of lecithin of known concentration were introduced into weighed 5 ml ampoules and evaporated in a stream of nitrogen and afterwards *in vacuo* to constant weight. Thereafter the ethanolic solutions of sodium palmitate were pipetted and again evaporated to dryness. Very finely powdered cholesterol was introduced through a small funnel, the amounts of it, being a 50 % approx. in excess of the amounts established in preliminary experiments. Finally, 2 ml of 100 millimolar sodium taurodeoxycholate in 0.15 M phosphate buffer *p*H = 7.3 were introduced and the ampoules sealed under nitrogen. Amounts of lecithin were calculated to give final concentrations in the sealed ampoules ranging between zero and 40 millimolar.

The ampoules were shaken using a Microid Flask Shaker (from Griffin and George Ltd., England) in a room constantly held at 37° C. After shaking for 4 days, the ampoules were left standing for three hours, whereafter the content was filtered (at 37° C) through Millipore fil-

ters, pore size 0.22 (from Millipore Filter Corp., Bedford Mass. USA) linked to 3 ml syringes.

For each system with identical conditions of bile salt, lecithin and sodium palmitate, usually 4 ampoules were prepared and three determinations of cholesterol were made in the filtrate from each ampoule.

Analysis of cholesterol. Aliquots of 300 and 500 microlitres of the filtrates were taken and its cholesterol analyzed, by the method described by HEGARDT and DAM (8) i.e. saponification with KOH, extraction with ether of the non saponifiable fraction and quantification of the colour formed by the Liebermann Burchard reaction at 625 nm, comparing the obtained figures with those of a standard of pure cholesterol.

Two issues were confronted to explore the effect in the solubilization of cholesterol, produced by the sodium palmitate when is introduced in the *in vitro* system previously used by HEGARDT and DAM (8): First, with a fixed concentration of sodium palmitate the concentration of lecithin was varied; second, the lecithin concentration was fixed and the amount of sodium palmitate varied from zero to 25 mM/l; this experiment was repeated at three different concentrations of lecithin; the dissolved cholesterol was then measured, in both sets of experiments.

Results

First experiment gave the results shown in figure 1. On the ordinate is represented the molar ratio of lecithin to cholesterol, on abscissa the molar ratio bile salt to cholesterol, according to the bilinear ratio graphing technic previously used by HEGARDT and DAM (8). The lower line represents the limit of saturation of cholesterol under the conditions described, 100 millimolar sodium taurodeoxycholate, variable amounts of egg lecithin between

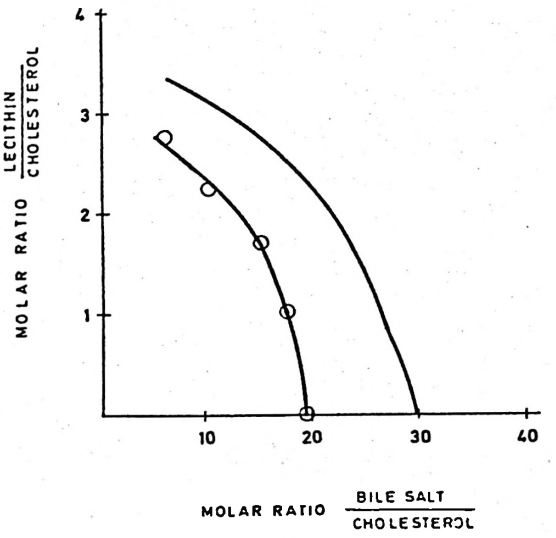

Fig. 1. *Curves indicating the limit for solubility of cholesterol in 100 millimolar aqueous solutions of sodium taurodeoxycholate, to which have been added amounts of chromatographycally purified egg yolk lecithin varying from zero to 40 millimoles per 100 mM of bile salt.*

Upper curve: No sodium palmitate has been added [data of HEGARDT and DAM, (8)]. *Lower curve:* 25 mM/l has been added. The ordinate represents the molar ratio of lecithin to cholesterol; the abscissa, the molar ratio bile salt to cholesterol.

0-40 mM/l and 25 mM/l of sodium palmitate. The upper line used as a standard for comparison, represents the saturation limit of cholesterol in the model system shown in the absence of sodium palmitate. This line previously reported by HEGARDT and DAM (8), was repeated by us, and results coincided.

From these results it can be deduced, that if to the *in vitro* model used by many authors, viz. bile salt:lecithin:water, 25 mM/l of sodium palmitate is added, the cholesterol dissolving capacity appreciably increases. This fact suggests that an *in vitro* or *in vivo* model may appear to be supersaturated (i.e. the point lies below and to the left of the upper line in figure 1)

and in fact, is not, provided that the concentration of sodium palmitate (a quantity hitherto ignored) has high values. In other words, all those points on the plane situated between the two lines can mislead if the content of sodium palmitate is not taken into consideration since according to the *in vitro* bile models formerly cited they would represent supersaturation of cholesterol, while in the presence of 25 millimolar of sodium palmitate they represent unsaturated solutions.

The second experiment which complements the first, was done preparing several series of ampoules, in every one of which a certain amount of lecithin was maintained and the proportion of sodium palmitate was varied from zero to 25 mM/l. This concentration was not exceeded not only because of its irrelevance to physiological conditions, but because of the increasing difficulty of filtration through the Millipore filter, owing to the formation of non-isotropic solutions, a concept which is described by SMALL *et al.* (21).

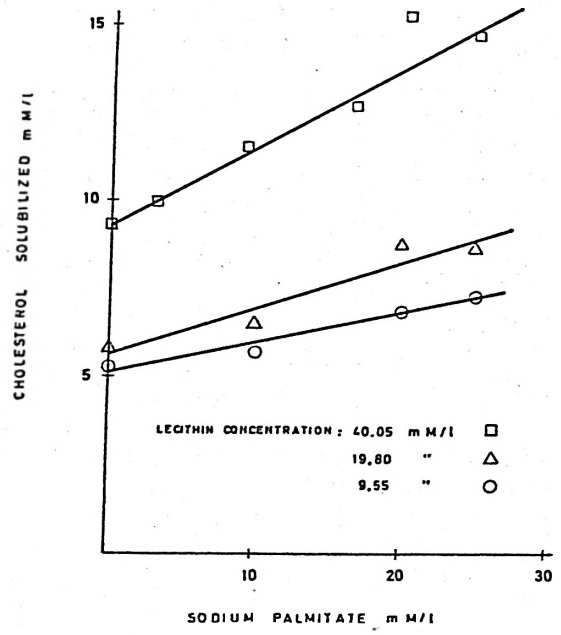

Fig. 2. *Straight lines representing the concentration of dissolved cholesterol in systems in vitro formed by 100 millimolar aqueous sodium taurodeoxycholate and fixed concentrations of egg yolk lecithin for each line. Ordinate:* millimolar concentration of solubilized cholesterol. *Abscissa:* millimolar concentration of sodium palmitate.

Table II. *Values of the regression coefficients, and the intercepts of the straight lines relating the amount of cholesterol dissolved in a model system of bile, with sodium taurodeoxycholate as the only bile salt, to the sodium palmitate concentration for three different concentrations of lecithin.*

Lecithin concentration mM/l	Slope × 10^2	Intercept	Correlation coefficient
9.55	8.57	5.12	0.9844
19.80	13.06	5.65	0.9603
40.05	22.32	9.35	0.8996

Figure 2 shows the results from these experiments. On the ordinate is represented the millimolar concentration of solubilized cholesterol, on the abscissa the millimolar concentration of sodium palmitate. In Table II are given the sample estimates of the intercept, slopes and correlation coefficients according to FREUND (7).

From the results, it is concluded that at increasing concentrations of sodium palmitate, more cholesterol goes into solution; for every series of fixed concentration of lecithin, the regression is lineal. Also it might be noted, that the slopes of the straight lines increase with the concentrations of lecithin. This interrelation is represented in figure 3 in which in ordinate are represented the slopes of the figure 2 multiplied by 100, and in abscissa the concentrations of lecithin. A straight line can be adjusted of which the estimates of the parameters are: intercept 0.042 and the slope 0.0045. The sample coefficient of correlation is 0.99994.

The data on the solubilizing power of

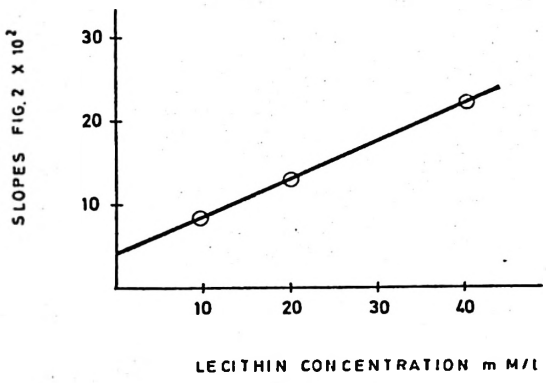

Fig. 3. *Regression line of the slopes of the straingt lines shown in figure 2 and the lecithin concentration.*
Ordinate: Slopes of the lines in figure 2 multiplied by 10^2. *Abscissa:* Concentrations of lecithin in mM/l.

sodium palmitate in the presence of lecithin, can be represented by the equation:

$$I = P\,(0.0045\,L + 0.042)$$

where in mM/l: I = Increase in cholesterol dissolved due to palmitate; P = Concentration of sodium palmitate, and L = Concentration of lecithin.

From it, can be calculated the excess of solubilized cholesterol attributable to palmitate for any specified value of lecithin comprised between zero and 40 millimolar.

Discussion

The free fatty acids, in small proportions, are normal components of the gallbladder bile (11). Their presence has been accounted for in two ways, either that they are directly secreted by hepatic bile, or by the fact that they are formed as a consequence of the hydrolytic degradation of the biliar lecithin (23, 24). It is known that a reflux of pancreatic secretions into the biliary tract could be produced in healthy humans and that these secretions have phospholipase activity, capable of splitting the lecithin molecule, originating free fatty acids, glycerides and lysolecithin. As a contribution to this assertion, NAKAYAMA *et al.* (15) found that small proportions of fatty acids could be found in pooled samples of normal bladder biles, these amounts being appreciably increased in people with gallbladder disfunctions (16).

It is known also, that the three major fatty acids components of human biliary lecithin are palmitic, linoleic and oleic, amounting to 80 % of the total fatty acids. The first two acids are predominant (5), although some authors have reported that oleic acid is more abundant than linoleic (2), as usually is the case in egg yolk lecithin (8) (Table I).

The effect of sodium oleate in the solubilization of cholesterol in a bile salt lecithin system has been recently reported by INOUE *et al.* (13) in an attempt to explain the occurrence of gallstones in biles whose composition can be profoundly changed by *Clostridium welchii* or other bacteria with phospholipase activity. Their results show that in some cases sodium oleate can help to solubilize cholesterol, although in most of them, the effect was to diminish its solubilization. It seems that most of the concentrations of oleate used were not at the physiological ranges, reported up to now (11, 15, 16).

We focused our studies on the possible influence of sodium palmitate, which is the most abundant fatty acid soap found in the bile. We were interested in studying the influence of sodium palmitate at physiological concentrations, trying to find an explanation for the high incidence of healthy people with supersaturation of cholesterol and no gallstones (4, 12). We chose sodium taurodeoxycholate because this is a bile salt whose solubilizing power is almost equal, at physiological concentrations of lecithin, to that of the pooled bile salts found in normal healthy people (8).

Our results show clearly that at low

concentrations of fatty acid similar to those found in the bile, sodium palmitate can facilitate the solubilization of cholesterol. We could speculate that decreases in the palmitate concentration can lead to supersaturation of a bile which previously was within the limits of saturation under relatively high amounts of sodium palmitate. Furthermore, our data could help to explain the apparent discrepancy between the presence of bile which is supposedly supersaturated and the absence of gallstones in humans.

We believe that palmitate may be one of the «forgotten water soluble components» conjectured to exist by MUFSON *et al.* (14). Further, we think that this study represents a closer approximation to the phenomenon of the solution of cholesterol in bile. However, we do not claim that the problem has been completely elucidated.

Two steps are usually considered as participating in the formation of cholesterol-rich gallstones. First, the real supersaturation of cholesterol in the bile system and consequently its precipitation as microcristals. Second, the enlargement of these cristals, trapping besides inorganic matter and other substances. A lot of research must be done towards the elucidation of the second step, which is probably the phenomenon of gallstone formation which at the present remains less known. However, we believe that the supersaturation of cholesterol in bile must be considered in its complete entity. Most of the work done, takes into account the described system, viz. bile salt:lecithin: water, considering either that there is no other substances in the bile with cholesterol solubilizing capacity or that the concentrations of these overlooked substances would be so small that their effect would be minimal. This approach may be an over-simplification and the supersaturation considered up to now becomes a good approximation. The present paper is a contribution in the sense that it is necessary to study the individual influence of the cholesterol solubilization of each of the substances which are in the bile, i.e. the phospholipids other than lecithin, the glycerides and the free fatty acids. The supersaturation of cholesterol described by several authors would be, perhaps misleading if experiments with these or other lipids were not made.

Resumen

Se ha ensayado el poder solubilizante de colesterol del palmitato sódico en modelos *in vitro* formados por taurodeoxicolato sódico y lecitina. De los resultados obtenidos se deduce que el palmitato sódico aumenta moderadamente el poder solubilizante de colesterol del sistema, y que la correlación existente entre el colesterol solubilizado y la concentración de palmitato es lineal, dependiendo la pendiente de la concentración de lecitina en el medio. Se ha calculado, por mínimos cuadrados una ecuación que relaciona los aumentos de colesterol debidos a la presencia del palmitato, y las concentraciones de esta sal y de lecitina.

Se sugiere que las discrepancias de comprensión entre sobresaturación de colesterol en bilis y ausencia de cálculos biliares, descritas en la literatura, pueden ser debidas a no haber tenido en cuenta las concentraciones de palmitato sódico en las bilis bajo estudio.

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