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The solubility of cholesterol in aqueous solutions of bile salts and lecithin

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With 3 figures and 1 table

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It is a well established fact that phospholipids and bile salts are of primary importance to the solubility of cholesterol in bile (SPANNER & BAUMAN (1)), (ISAKSSON (2)).

In recent years, thorough physico-chemical studies of the system water-lecithin-bile salt-cholesterol have been carried out by SMALL, BOURGÉS & DERVICHIAN (3), and the detergent properties of bile salts have been extensively reviewed by HOFMANN & SMALL (4).

Further, studies of the solubility of cholesterol in aqueous solutions of lecithin and bile salts have been presented by NEIDERHISER & ROTH (5) and by SAUNDERS & WELLS (6).

Independently, work on this latter subject has been carried out in our laboratory and is still in progress. Here we are reporting the present results of this work. A subsequent paper will deal with the application of the results to the data for composition of bladder biles from experimental animals and man previously reported from this laboratory.

Material and methods

Bile salts

Sodium taurocholate (Calbiochem), sodium taurodeoxycholate (Calbiochem) and sodium taurochenodeoxycholate (Sigma) were tested for purity by thin-layer-chromatography of 200 microgram bile salt on silicagel H; (moving phase: benzene: ethanol:acetic acid 30:10:2) and found pure. Further quantities of sodium taurocholate and sodium taurodeoxycholate were prepared in our laboratory by the method of NORMAN (7) and purified according to HOFMANN (8). The unconjugated cholic acid and deoxycholic acids used as starting materials were obtained from Mann Research Laboratories, purified by crystallization and tested for purity before they were conjugated. Taurine was obtained from Hoffmann-La Roche & Co., Basel.

Sodium glycocholate, sodium glycodeoxycholate and sodium glycochenodeoxycholate were prepared in our laboratory starting from the corresponding unconjugated bile acids obtained from Mann Research Laboratory, and glycine from Merck, Darmstadt. Glycocholate and glycodeoxycholate were prepared by the method of NORMAN (7) and purified by the method of HOFMANN (8). Glycochenodeoxycholate was prepared and purified as described by HOFMANN (9). The purity of the glycine-conjugated bile salts was tested by thin-layer-

chromatography as described for the taurine-conjugated bile acids. Glycocholate and glycodeoxycholate were found to be pure, but glycochenodeoxycholate contained a small amount of an impurity migrating as glycocholic acid.

The percentage of moisture (weight loss at 105 °C) was determined for all the bile salts prepared.

Cholesterol:

Cholesterol, from Glaxo Laboratories Ltd., was recrystallized twice from ethanol. Thin layer chromatography on silicagel H (moving phase benzene:ethyl acetate:ether:acetic acid 80:10:10:0.2) did not reveal presence of impurities.

Lecithins:

1. Commercial Lecithin *ex ovo*, obtained from Northern Drug- and Chemical Company, Copenhagen through a Pharmacy, was purified on columns of Aluminum oxide, 250 g for 10 g crude lecithin, as described by Singleton et al. (10).

The starting material contained a considerable amount of impurities. For every 10 g of the crude product, the column was washed first with 300 ml chloroform, thereafter in sucession with 100 ml of chloroform:methanol 30:1, 100 ml of chloroform:methanol 20:1, and 700 ml of chloroform:methanol 9:1.

The last 700 ml effluent from the column was collected in fractions of 50 ml each. Each fraction was examined by thin-layer chromatography (moving phase chloroform:methanol: water 100:40:6). The fractions containing only small amounts of lysolecithin were combined, evaporated to dryness, dissolved in a suitable amount of chloroform and purified again on columns of aluminum oxide by the same method.

The final product was tested by thin layer chromatography on silicagel H (moving phase chloroform:methanol:water 100:40:6) and was found to be pure. The yield of pure lecithin was about 37% of the weight of the crude lecithin.

2. Two other egg lecithins were prepared from two different samples of lyophilized egg yolks, furnished by dr. C. C. CALVERT, United States Department of Argiculture, Poultry Research Branch, Beltsville, MD., through dr. M. L. SCOTT, Cornell University, Ithaca. N. Y.

One of the samples of egg yolk originated from hens that had received no polyunsaturated fatty acids whereas the other originated from hens that had received 15% safflower oil in their ration during the laying period.

The procedure for preparing lecithin from egg yolks was as follows:

40 g of the lyophilized egg yolk was extracted with 200 ml acetone three times and the extracts discarded. The undissolved portion was extracted with 400 ml of chloroform: methanol 9:1 three times and the combined extracts evaporated to dryness.

The dry residue containing the crude lecithin was subjected to purification on aluminum oxide as already described.

The yield of pure lecithins was between 11 and 12% of the weight of the lyophilized egg yolks.

3. *Lecithin from human bile*

Bladder bile from peptic ulcer patients not having diseases of the biliary tract served as starting material.

Portions of about 5 ml bile were extracted with 100 ml chloroform:methanol 2:1. After filtering, the extract was shaken with one fitfh of its volume of 0.73% aqueous NaCl solution and centrifuged. After removal of the aqueous phase, the organic phase was washed 4 times with a mixture consisting of chloroform:methanol :0.73% NaCl solution 3:48:47 (v:v:v), the volume of the mixture used for each washing being equal to the volume of the aqueous phase removed. The washed non-aqueous phase was evaporated to dryness and dissolved in 5 ml chloroform.

The chloroform solution was applied to a column of 15 g Silicic acid (Baker) and 5 g Hyflo-Super-Cel. The non-phosphatidic lipids were eluted with (at least) 200 ml chloroform. Thereafter the phosphatides were eluted with (at least) 150 ml methanol.

The combined methanol solutions from 4 such preparations were evaporated to dryness, dissolved in a small volume of chloroform and applied to a column of 40 g aluminum oxide washed with chloroform. Subsequently, the phosphatides were eluted with 700 ml of chloroform:methanol 9:1, the effluent solution being collected in fractions of 50 ml each. Each fraction was tested by thin-layer chromatography as described in under purification of egg lecithin. Those fractions showing the presence of lecithin free from impurities were combined, evaporated to dryness, dissolved in a few ml of chloroform and stored in sealed ampoules under nitrogen at minus 20 °C until use. The yield of pure lecithin varied greatly for different bile samples.

The fatty acid composition of the different lecithins shown in table 1 was determined by gas-liquid-chromatography as previously described (11).

Determination of the solubility of cholesterol

Chloroform solutions of lecithin of known concentration were pipetted into weighed 5 ml ampoules and evaporated in a stream of nitrogen and afterwards *in vacuo* to constant weight. Thereafter 2 ml of a solution of the bile salt in 100 millimolar phosphate buffer of pH 7.3 was introduced. In most cases the concentration of the bile salt was 100 millimolar, but in some experiments concentrations of 50 and 200 millimolar were also used. The amounts of lecithin were chosen so as to provide molar ratios between bile salt and lecithin of approximately 100:80, 100:40, 100:20, 100:10, 100:5 and 100:0. Finely powdered cholesterol was introduced through a small funnel having a suitably tapered tube, whereafter the ampoules were sealed in a stream of nitrogen. In most cases, the amounts of cholesterol introduced were 50 per cent in excess of the amount corresponding to the approximate solubility established in preliminary experiments, but in case of the lowest ratio between bile salt and lecithin only a 20 per cent excess was used.

The sealed ampoules were shaken in a room constantly held at 37 °C, using a Microid Flask Shaker (from Griffin & George Ltd., England). After shaking for 4 days, the ampoules were left standing at 37 °C for 2 days whereafter the content was filtered (at 37 °C) through Millipore Filters, pore size 0.22 μ (from Millipore Filter Corporation, Bedford, Mass., U.S.A.). All the filtrates used for the subsequent determination of cholesterol were clear.

For each system with identical ratio between bile salt and lecithin, usually 4 ampoules were prepared, and 3 or 4 determinations of cholesterol were made in the filtrate from each ampoule. In the case of taurodeoxycholate without lecithin 12 ampoules were prepared.

Aliquots of 200, 300, 400 or 500 microlitres of the filtrates (according to the expected concentration of cholesterol) were heated on steam bath with 1 ml 30% KOH (w:v) for 5 minutes. After cooling and dilution with water, the non-saponifiable fraction was shaken out with ether, washed with alkaline water and distilled water, whereafter the ether solution was evaporated to dryness. The residue was dissolved in chloroform and the solution diluted to 25 ml in a measuring flask. A measured volume, usually 10 ml, was evaporated to dryness, dissolved in 5 ml chloroform, whereafter the LIEBERMANN-BURCHARD reaction was carried out by addition of 1 ml of the reagent (20 ml acetic anhydride + 2 ml concentrated sulfuric acid). After a reaction time of 20 minutes at 25 °C, protected from light, the absorbance at 625 nm was measured and compared with the absorbance produced by a standard solution of pure cholesterol.

Double determinations of cholesterol in one and the same filtrate rarely differ more than 5% from each other, but from one ampoule to another with supposedly identical content the difference can be greater. Apparently, some errors are due to incomplete saturation. Therefore, instead of taking the mean value of the cholesterol concentrations in all of a group of ampoules and to calculate standard errors therefrom, it may be safer to exclude possible particularly low values and to attribute an arbitrary confidence limit of 10% to the mean value of the others.

Our method for determination of the solubility resembles that used by NEIDERHISER & ROTH (5) but whereas these investigators determined both cholesterol and bile salts in the filtrate we only determine cholesterol and assume that lecithin and bile salts still are in solution. This assumption is believed to hold at least for ratios between lecithin and bile salts up to 40:100 (the undissolved particles were only ordinary cholesterol crystals, and millipore filtration was found not to lower the concentration of dissolved cholesterol). With ratios between lecithin and bile salts 80:100, excess of cholesterol may be pressent not only in the ordinary crystalline form but also in the form of liquid crystals containing other components (3,4). The results obtained with this ratio, therefore, are less certain than the other results.

It is convenient to express the results in terms of the molar ratios between lecithin and cholesterol and between bile salt and cholesterol, and to plot the results graphically as in fig. 1. Strictly speaking, this requires a calculation of the concentrations of lecithin and bile salt in the final solution using a correction for the increase of volume caused by the uptake of lecithin and cholesterol into the two ml bile salt solution. Originally, we corrected the volume by determining the specific gravity before and after uptake of lecithin and cholesterol. It is sufficient, however, merely to add the volume of the lecithin which is its weight in mg divided by its specific gravity 1.03 (reference 12). For a 100 millimolar solution of the bile salt the increase in volume is near 3.5% when the amount of lecithin is 40 mM, and near 7% when the amount of lecithin is 80 mM. The small change in volume caused by the uptake of cholesterol can be neglected.

Results and Discussion

100 millimolar bile salts

Fig. 1 shows the limits for saturation with cholesterol for 100 millimolar solutions of the sodium salts of Glycodeoxycholic acid (GD), Taurodeoxycholic acid (TD), Glycochenodeoxycholic acid (GCD), Taurochenodeoxycholic acid (TCD), Glycocholic acid (GC), and Taurocholic acid (TC), to which is added lecithin purified from commercial egg lecithin in amounts varying from zero to approximately 80 mM per 100 mM bile salt.

For each bile salt, the solubility limit for cholesterol is represented by a curve indicating the molar ratios bile salt to cholesterol as abscissa and the molar ratios lecithin to cholesterol as ordinate.

The ability to dissolve cholesterol decreases from GD to TC. The greatest differences between the different bile salts with respect to cholesterol dissolving capacity occur when no lecithin is present. The differences decrease as the concentration of lecithin increases and eventually becomes the dominant factor.

When 40 mM of lecithin were present for each 100 mM of bile salt the point representing GC was still found to be located higher than the points representing TD and GD, but the method is not accurate enough to indicate the exact order of succes-

sion of the points for all the bile salts in this part of the diagram. Here the curves have been drawn in continuation of those found with lower ratios lecithin to bile salt in accordance with the evident tendancy to curvature and convergence. The curves cannot be prolonged to contact with the vertical axis, however, since this would require an infinite ratio between lecithin and bile salt. Furthermore, when the ratio between lecithin and bile salt increases beyond a certain value the system may form 3 phases as shown by SMALL et al. (3), and at still higher values it may become turbid even in the absence of cholesterol. With the assumed confidence limit of 10%, all the differences between the starting points on the horizontal axis are considered significant except the difference between TD and GCD, and the difference between TCD and GC.

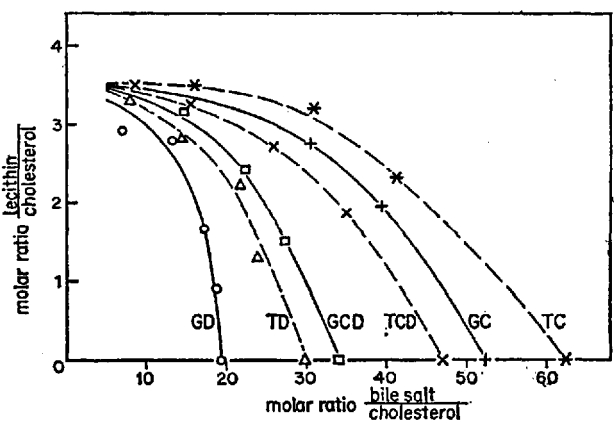

Fig. 1. Curves indicating limits for solubility of cholesterol in 100 millimolar aqueous solutions of the sodium salts of each of the six bile acids: Glycodeoxycholic (GD), Taurodeoxycholic (TD), Glycochenodeoxycholic (GCD), Taurochenodeoxycholic (TCD), Glycocholic (GC), and Taurocholic (TC), to which were added amounts of chromatographically purified egg lecithin varying from zero to approximately 80 millimoles per 100 millimoles bile salt.
Abscissa: molar ratio bile salt to cholesterol. Ordinate: molar ratio lecithin to cholesterol.

50 millimolar and 200 millimolar bile salt solution

Using sodium taurodeoxycholate (TD) and the same phosphate buffer as before, the solubility of cholesterol has been determined also with 200 millimolar and with 50 millimolar solutions of bile salt to which lecithin purified from commercial egg lecithin was added in the same approximate ratios as before. Compared to the curve obtained with 100 millimolar TD, no certain changes were found.

Two different egg lecithins

With 100 millimolar solutions of TD and phosphate buffer as before, the solubility of cholesterol was determined using lecithin purified from egg yolks from hens that had received no safflower oil in their ration (and almost no polyunsaturated fatty acids), and lecithin purified from egg yolks of hens that had received 15% safflower oil in their diet. In spite of the greatly different fatty acid composition of these two lecithins (table 1), no difference between their ability to dissolve cholesterol could be established with certainty.

Table 1. Fatty acid composition of chromatographically purified lecithins

	Source: Commercial Egg Lecithin	Source: Yolk of Hens fed 0% Safflower oil	Source: Yolk of Hens fed 15% Safflower oil	Source: Human Bile
14:0	0.2	0.9	0.8	0.5
16:0	29.9	29.4	24.2	36.7
16:1 ω 7	2.2	4.3	0.7	4.4
16:2				1.5
18:0	12.7	11.4	18.4	3.8
18:1 ω 9	29.4	46.9	15.6	11.2
18:2 ω 6	15.4	1.0	31.0	25.3
18:3 ω 3				1.0
20:3 ω 9		5.3		
20:3 ω 6				1.6
20:4 ω 6	4.1	0.2	7.3	7.3
20:5 ω 3	0.4			2.2
22:4 ω 6	0.4		0.4	
22:5 ω 6	0.8		1.4	
22:6 ω 3	4.4	0.2	0.1	4.6

Mixed bile salts as in human bile and human bile lecithin

Determinations of the solubility of cholesterol was also carried out with a 100 millimolar solution of total bile salts of a composition corresponding to that occurring in human bile to which was added lecithin from human bile in concentrations similar to those used in the foregoing experiments with purified egg lecithins.

The molar percentages of the individual bile salts in the total bile salts were as follows: GC 26.1, GCD 21.7, GD 15.7, TC 14.5, TCD 12.8, TD 9.2. These molar percentages are near the mean values for the molar percentages found in human duodenal bile from healthy young volunteers, in human bladder bile from peptic ulcer cases free from diseases of liver and biliary tract and in human bladder bile from cholelithiasis cases having functioning gall bladder. (reference 13). (In reference 13, TCD and TD are not presented separately from each other, therefore their sum TCD + TD has arbitrarily been divided into TCD and TD under the assumption that the ratio between TCD and TD is identical with the ratio between GCD and GD).

The result is shown in fig. 2, in which also the data for the solubility of cholesterol in a 100 millimolar solution of the sodium salt of GCD and lecithin purified from commercial egg lecithin are plotted. It is seen that the curve for mixed bile salts and human bile lecithin is practically identical with that for GCD and purified egg lecithin.

The cholesterol dissolving capacities of the six different bile salts seem to be additive, at least with a fair degree of approximation. Under this assumption, the number of millimoles of cholesterol which can be held in solution by 100 millimoles of bile salts of the just mentioned composition when no lecithin is present is

$$\frac{26{,}1}{52}+\frac{21.7}{34}+\frac{15.7}{19}+\frac{14.5}{62}+\frac{12.8}{47}+\frac{9.2}{30}=2.78$$

(The divisors are the distances on the horizontal axis from zero to the starting points of the curves representing the individual bile salts).

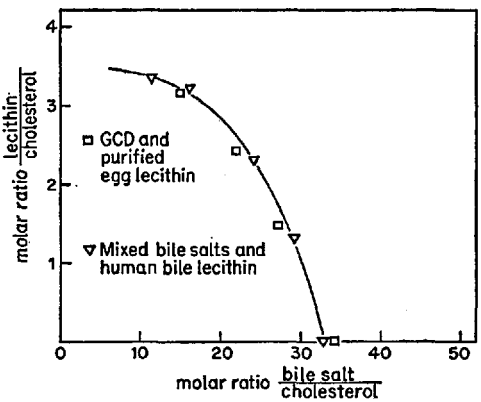

Fig. 2. Curve indicating the limit for solubility of cholesterol in a 100 millimolar aqueous solution of a mixture of the sodium salts of the six bile acids: GD, TD, GCD, TCD, GC and TC to which are added amounts of lecithin from human bile varying from zero to approximately 80 millimoles per 100 millimoles of total bile salts. The molar percentages of the individual bile salts in the total bile salts correspond to those found in human bile.

Points marked ▽ refer to the bile salt mixture with human bile lecithin; points marked □ refer to GCD and purified egg lecithin.

For abbreviations see legend to fig. 1.

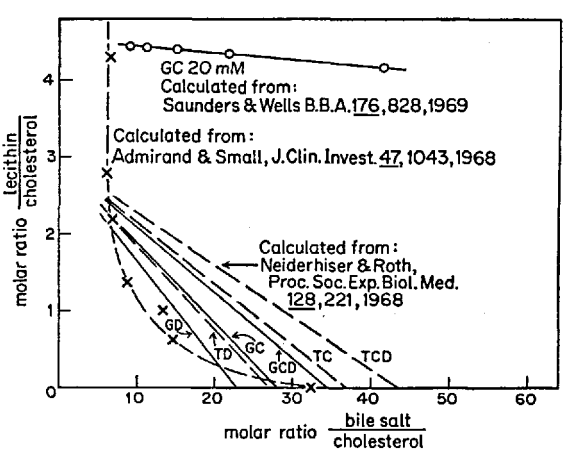

Fig. 3. Limits for solubility of cholesterol in aqueous solutions of bile salts and lecithin based upon data from three different laboratories.

Abscissa: molar ratio bile salts to cholesterol. Ordinate: molar ratio lecithin to cholesterol.

For abbreviations see legend to fig. 1.

The ratio total bile salts to cholesterol will then be

$$\frac{100}{2{,}78} = 36$$

which is in fairly good agreement with the experimentally determined starting point 34 for the curve representing the mixture of the bile salts.

The concentrations of inorganic cations in our system with 100 mM bile salts (before addition of lecithin) are Na^+225 mEq and K^+ 37,5 mEq. The corresponding figures for normal human bladder bile indicated by LARGE et al. (14) are in mEq: Na^+ maximal 240, average 200, minimal 100; K^+ maximal 16,7 average 12,7, minimal 6,3. Further, normal human bladder bile contains an average concentration of 20 mEq Ca^{++} according to the same authors. Examination of the influence of varying concentrations of inorganic ions on the solubility of cholesterol as well as the influence of higher or lower concentrations of bile salts than those used in the present study will require further experimentation.

It is of some interest to compare our results with those of NEIDERHISER & ROTH (5) and of SAUNDERS & WELLS (6).

The first mentioned authors have suggested equations from which the concentration of cholesterol at the saturation point can be calculated when the concentrations of lecithin and the bile salts in question are given. When the results for each of the 6 bile salts we are dealing with are calculated from these equations and plotted graphically as in fig. 3 they appear as straight lines directed towards the point 2,94 on the vertical axis, whereas our results appear as curved lines directed towards the point 3,5 (approximately). The sequence from left to right on the horizontal axis is GD, TD, GC, GCD, TC, TCD, whereas according to our results the sequence is GD, TD, GCD, TCD, GC, TC. Nevertheless, the findings of Neiderhiser & Roth agree with our findings in the following respects: Among the bile salts in question, that of GD is the most efficient solubilizer for cholesterol, and generally, the salts of the glycine-conjugated bile acids are better solubilizers for cholesterol than are the salts of the corresponding taurine-conjugated bile acids. A mixture of the six bile salts analogous to that of human bile has a solubilization capacity for cholesterol almost identical with that of the salt of GCD. Further: the differences in solubilizing capacity are greatest on the horizontal axis and diminish as the concentration of lecithin increases.

Based on experiments with 20 millimolar solutions of sodium glycocholate and a method (sonication and measurement of turbidity) which is different from ours, SAUNDERS & WELLS have presented an equation for calculation of the concentration of cholesterol at the saturation point from the concentrations of glycocholate and lecithin. When plotted as in fig. 3, the results appear as a straight line intersecting the vertical axis at the point 4,5 and the horizontal axis at the point 500. These results do not agree with ours nor with those of NEIDERHISER & ROTH.

Summary

The solubility of cholesterol in aqueous solutions of bile salts and lecithin with $\frac{N}{10}$ phosphate buffer, pH 7.3 has been examined.

For the sodium salts of the six bile acids used in the study, the capacity for dissolving cholesterol was found to decrease in the following order: Glycodeoxycholate, Taurodeoxycholate, Glycochenodeoxycholate, Taurochenodeoxycholate, Glycocholate, Taurocholate.

The differences with respect to cholesterol dissolving capacity were greatest when no lecithin was present, and decreased as the amount of lecithin increased.

Graphical representations (molar ratio bile salt to cholesterol as abscissa, molar ratio lecithin to cholesterol as ordinate) of the limits for solubility of cholesterol in 100 millimolar solutions of each of the six bile salts to which purified egg lecithin is added in amounts varying from zero to approximately 80 millimoles per 100 millimoles bile salt, had the shape of curved lines tending to converge against a point, approximately 3.5, on the vertical axis but without reaching it. (Fig. 1). The starting points of the curves on the horizontal axis (millimoles bile salt required to dissolve 1 millimole of cholesterol in the absence of lecithin) were: for Glycodeoxycholate 19, for Taurodeoxycholate 30, for Glycochenodeoxycholate 34, for Taurochenodeoxycholate 47, for Glycocholate 52, for Taurocholate 62.

The graphical representation, as above, of the limit for solubility of cholesterol in a 100 millimolar solution of a mixture of the six bile salts in relative amounts as in human bile, to which lecithin from human bile is added was practically identical to the corresponding curve for a 100 millimolar solution of glycochenodeoxycholate with additions of purified egg lecithin (Fig. 2.).

The starting point on the horizontal axis for the curve representing mixed bile salts could be calculated – with fair approximation – from the cholesterol dissolving capacities of the individual bile salts in the mixture.

The graphical representations of the limits for solubility of cholesterol in 50 millimolar, 100 millimolar and 200 millimolar aqueous solutions of sodium taurodeoxycholate to which purified egg lecithin was added in varying amounts did not show any certain differences from each other.

Two purified egg lecithins of greatly different fatty acid pattern (Table 1) isolated from eggs of hens fed linoleic acid deficient diets and linoleic acid rich diets respectively, did not show any certain difference from each other with respect to cholesterol dissolving capacity when added in varying amounts to a 100 millimolar solution of sodium taurodeoxycholate.

Zusammenfassung

Die Löslichkeit von Cholesterin in wässerigen Lösungen von Gallensäuresalzen und Lecithin, mit $\frac{N}{10}$ Phosphat-Puffer pH 7.3, wurde untersucht.

Unter den Natriumsalzen der sechs untersuchten Gallensäuren hatte Glycodeoxycholat das höchste Lösungs-Vermögen für Cholesterin. Davon nahm das Lösungs-Vermögen ab in folgender Reihenfolge: Taurodeoxycholat, Glycochenodeoxycholat, Taurochenodeoxycholat, Glycocholat, Taurocholat.

Die Unterschiede in Bezug auf das Cholesterin-Lösungsvermögen waren am größten, wenn die Lösungen kein Lecithin enthielten, sie nahmen ab mit zunehmendem Lecithin-Gehalt.

Graphische Darstellungen (Moläres Verhältnis Gallensäuresalz/Cholesterin als Abscisse, Moläres Verhältnis Lecithin/Cholesterin als Ordinat) der Sättigungs-Grenzen für Cholesterin in 100 millimolären Lösungen jeder der sechs untersuchten Gallensäuresalzen zu welchen chromatographisch gereinigtes Ei-Lecithin in Quantitäten von Null bis ungefähr 80 Millimol per 100 Millimol Gallensäuresalz zugesetzt wurde, erschienen als krumme Linien mit Richtung gegen einen Punkt, ungefähr 3.5, auf der Ordinate, ohne jedoch diesen Punkt zu erreichen (Fig. 1). Die Anfangspunkte der Kurven auf der Horizontalachse (Anzahl Millimol des betreffenden Gallensäuresalz, die nötig sind um 1 Millimol Cholesterin in Lösung zu halten in Abwesenheit von Lecithin) waren: für Glycodeoxycholat 19, für Taurodeoxycholat 30, für Glycochenodeoxycholat 34, für Taurochenodeoxycholat 47, für Glycocholat 52, für Taurocholat 62.

Die graphische Darstellung der Sättigungs-Grenze für Cholesterin in einer 100 millimolären Lösung einer Mischung der sechs Gallensäuresalzen, in Mengenverhältnissen wie in menschlicher Galle, wozu Lecithin aus menschlicher Galle in variierender Menge zugesetzt

wurde, war mit der entsprechenden Darstellung der Sättigungs-Grenze für Cholesterin in 100 millimolarer Lösung von Natrium Glycochenodeoxycholat mit Zusatz von chromatographisch gereinigtem Ei-Lecithin praktisch zusammenfallend. (Fig. 2.). Der Anfangspunkt der für gemischte Gallensäuresalze geltenden Kurve auf der Horizontalachse ließ sich aus den Cholesterin-Lösungsvermögen der einzelnen Gallensäuresalzen mit befriedigender Annäherung berechnen.

Die graphischen Darstellungen der Sättigungs-Grenzen für Cholesterin in 50 millimolaren, 100 millimolaren und 200 millimolaren Lösungen von Natrium-Taurodeoxycholat mit variierendem Zusatz von chromatographisch gereinigtem Ei-Lecithin waren nicht mit Sicherheit verschieden von einander.

Zwei Ei-Lecithine von sehr verschiedenem Fettsäuremuster (Tabelle 1) isoliert aus Eiern von Hennen, welche mit linolsäuredeficienten bzw. linolsäurereichen Nahrungen gefüttert worden waren, zeigten keine sichere Verschiedenheit von einander in bezug auf Cholesterin-Lösungsvermögen, wenn dieselben in variierender Menge, einer 100 millimolaren Lösung von Natrium-Taurodeoxycholat zugesetzt wurden.

Acknowledgement

We thank Dr. C. C. CALVERT, United States Agricultural Research Service, Animal Husbandry Research Division, Beltsville, Maryland for the gift of lyophilized egg yolks from hens fed high and low linoleic acid rations.

Addendum

(Received August 4, 1970)

After having attended the Conference on Gallstone Problems held July 14th 1970 in Copenhagen in connection with the 4th World Congress of Gastroenterology we have given particular consideration to the data for solubility of cholesterol in aqueous solutions of bile salts and lecithin presented by ADMIRAND & SMALL (15). Part of a curve constructed from their data pertaining to a mixture of the six bile salts in question and lecithin is plotted in fig. 3 together with the curves based upon data determined and equations suggested by NEIDERHISER & ROTH (5) and by SAUNDERS & WELLS (6).

The curve based upon the data from ADMIRAND & SMALL (15) continues upwards beyond the frame of fig. 3, passes through a point having the coordinates bile salts to cholesterol = 14.2, lecithin to cholesterol = 17.7, and further on *ad infinitum* in both coordinates. In the original paper (15), the data were illustrated in triangular coordinates.

The data are valid for total concentrations of bile salts, lecithin and cholesterol varying from 5 to 20% by weight. Our system based on a 100 millimolar solution of mixed bile salts contains about 5% (w/v) bile salts plus cholesterol in the absence of lecithin, about 8.5%bile salt plus cholesterol plus lecithin when 40 mM lecithin is present, and about 12% bile salts plus cholesterol plus lecithin when 80 mM lecithin is present. It is, therefore, valid in a range of concentrations for which data of ADMIRAND & SMALL are assumed to hold.

Concerning the starting point of the curve for mixed bile salts on the horizontal axis (the molar ratio bile salt to cholesterol in absence of lecithin) there is good agreement between the value 32 found by ADMIRAND & SMALL and the value 34 found by NEIDERHISER & ROTH and by us.

There is also agreement about the fact that the curve does not touch the vertical axis.

But whereas we find a curve turning the convexity away from the zero, zero point, NEIDERHISER & ROTH find a straight line, and ADMIRAND & SMALL find a curve turning the convexity toward the zero, zero point.

The cause of these discrepancies will be examined later.

It should be emphasized that our method does not show what happens to the solubility of cholesterol at very high ratios between lecithin and bile salts. Thus, it cannot be excluded that further investigations by suitable methods may show an upward turn of the curve in this

particular region more or less like the upper part of the curve based on the results of ADMIRAND & SMALL. But this part of the curve refers to ratios that are not likely to be found in bile.

Concerning the choice of coordinate system the following considerations may be appropriate:

The triangular system is the obvious means for presenting the results when the purpose is to indicate the limits for occurrence of the different phases in an *in vitro* system in which the ratios between bile salt, lecithin and cholesterol can be varied at will, such as was the case in the elegant study of SMALL et al. (3).

But in bile from humans, hamsters and mice, the variations of these ratios are limited, and especially, the ratio between total bile salts and lecithin only varies between certain not very wide limits characteristic of the species in question. For comparison of the limit for solubility of cholesterol with the ratios found in bile, a two-dimensional system, therefore, is sufficient and more convenient to use.

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