# Application of Gel Filtration of Bile Acids to Studies of Lipid-Complexes in Bile.*† (29403)

ARNE NORMAN (Introduced by B. E. Gustafsson)
*Department of Clinical Chemistry, Stockholms Läns Centrallasarett, Danderyd, Sweden*

Several hypotheses for the physical state of lipids and bile acids in gall bladder bile have been published (cf. 1). Isaksson(2,3) has presented evidence for the existence of a water soluble complex between bile acids and phospholipids in bile. The lecithin bile salts system, the LBS system,‡ was considered to be capable of keeping cholesterol soluble in bile. In contrast Verschure(4) and Dessi and Franco(5) claimed that the lipids in bile are kept in water solution as lipoproteins. They employed paper electrophoresis and have isolated a high mobility complex, the bili-lipoprotein, which contained protein, cholesterol, phospholipids, bile acids and bilirubin. The present author has demonstrated (6) that the high mobility complex is rather unstable and can be easily decomposed by diluting the bile with saline. After dilution the proteins, bile acids, and cholesterol appeared in separate electrophoretic fractions. In an attempt to clarify the exact physical state of the bile acids in gall bladder bile, the present investigation was performed with gel filtration instead of paper electrophoresis.

*Experimental.* $C^{14}$ labeled bile acids were synthesized and purified as described earlier (7). 10 $\mu$c of the sodium salts of labeled bile acids were given orally to patients one day before elective cholecystectomy for cholelithiasis. Cholecystography had revealed a functioning gall bladder. Bile was obtained at operation by puncture of the gall bladder after ligation of the cystic duct. Neither the bile nor the gall bladder wall gave any gross indication of stasis or infection.

The Sephadex columns were prepared as described by Flodin(8) and Beling(9). The columns were 13 mm in diameter, and 350 mm in length, and were prepared by free sedimentation of the gel grains in a glass column. Reference bile acids were dissolved in 2.0 ml effluent or 0.5 ml gall bladder bile diluted with 1.5 ml of effluent. These solutions were allowed to percolate into the bed material. Effluent was passed through the columns at a rate of 0.2-0.3 ml per minute and was collected with the aid of a drop-counter into previously weighed tubes. The standardization of each column was made by carrying out gel filtration of R I S A‡ dissolved in 0.15 M potassium chloride.

After gel filtration of bile the labeled bile acids and the bile lipids remaining on the columns were extracted as follows: the gel was transferred from the column into a flask, dried in an oven at 100°C, and refluxed with chloroform:methanol 2:1 for 3 hours. Aliquots of the filtrate were analyzed.

The R I S A was determined by its $\gamma$-radiation and potassium ions by a flame photometric method. $C^{14}$ in the effluent was determined by plating aliquots and counting with a Frieseke-Hoepfner methane gas-flow counter. For quantitative measurement of bile lipids the effluent was extracted by a modified Folch extraction technic. The extraction was carried out at room temperature with 15 volumes of a mixture of chloroform:methanol 2:1. The extract was partitioned with an equal volume of saline, and the chloroform layer was separated. Following evaporation of the solvent, the residue was redissolved in chloroform, and aliquots were used for determination of cholesterol (10) and phospholipids(11).

*Results. Reference bile acids.* In each ex-

* Bile acids and steroids 148.

† This work is part of investigations supported by the Statens Medicinska Forskningsrad and Stockholms Läns Landsting.

‡ All labeled bile acids, free and conjugated, contained $C^{14}$ in carbon number 24. Abbreviations used are: GC, glycocholic acid; GD, glycodeoxycholic acid; GCD, glycochenodeoxycholic acid; GL, glycolithocholic acid; TC, taurocholic acid; TD, taurodeoxycholic acid; TCD, taurochenodeoxycholic acid; TL, taurolithocholic acid; LBS system, water soluble lecithin-bile salt complex(2); R I S A, radioiodinated serum albumin.

periment a single $C^{14}$ labeled bile acid was gel filtered through a standard Sephadex column. Ten to twenty $\mu$g of the sodium salt of each bile acid were dissolved in 2 ml of effluent and added to a column, which was then eluted with either water or saline. Elution curves obtained in experiments with GC‡, TC, GD, TD, GL and TL are shown in Fig. 1. In the upper part of Fig. 1 elution curves obtained for R I S A and potassium chloride are given as a reference. The results indicated that both molecular filtration and adsorption chromatography occurred during the procedure. The latter was more pronounced when the columns were eluted with saline than with water. The order of elution from the columns was: TC, GC, TD, GD, TL and GL. Although the difference in elution rate of TC and GC and that of TD and GD was not significant, GL was eluted more slowly from the columns than TL. Similar experiments were performed with TCD and GCD. There was no significant difference in the elution rate of GCD and TCD, which appeared in approximately the same fractions as GD and TD. The sodium salts of unconjugated bile acids, such as cholate, deoxycholate and chenodeoxycholate, were eluted at the same rate as the corresponding glycine or taurine conjugates.

*In vitro labeling of bile.* The same experiments as described above were performed with the reference labeled bile acids dissolved in gall bladder bile. The elution rates of TC, TD and TL from standard columns eluted with either water or saline are shown in Fig. 2. The elution curves of TC, TD, TCD, GC, GD and GCD were almost identical to those obtained for the reference bile acids (Fig. 1). No labeled compounds were found in the macromolecular fraction of bile and no isotope was appreciably retained by the columns. In contrast, however, TL and GL behaved differently. The major part of the labeled GL and TL dissolved in the bile was eluted from the column in the same way as labeled GL and TL dissolved in water. However, small amounts of isotope (2 to 6%) were always recovered in front of the main peak, closely associated with the macromolecular fraction. In addition, isotope (10 to 27%) was always retained by the columns. The percentage of isotope in these two fractions was generally higher when the gel filtration was performed with saline than with water.

*In vivo labeling of bile.* Ten $\mu$c of $C^{14}$ labeled cholic, chenodeoxycholic or lithocholic acid was administered orally to patients one day before their gall bladder bile was to be collected. The elution curves of the *in vivo* labeled bile are shown in Fig. 3. Practically identical results were obtained as with the bile labeled *in vitro*.

*Surface active compounds in bile.* The upper part of Fig. 2 gives the weights of each 32 drop fractions. By this means the presence of surface active compounds can be demonstrated. The main surface active compounds in bile are the bile acids. Their location in the effluent fractions was determined by this method. In the macromolecular fraction, surface active compounds were detected in almost all gel filtrations with saline but were nearly always absent in gel filtrations with water.

*Cholesterol and phospholipids.* The behavior of cholesterol and phospholipids during gel filtration of bile with either water or saline has been studied in 12 samples of gall bladder bile. In all gel filtrations cholesterol and phospholipids were eluted in the void volume, *i.e.* in the macromolecular fraction, clearly separated from the eluted bile acids (Fig. 2). The shape of the elution curves for cholesterol and phospholipids were always identical. However, complete recovery of added cholesterol and phospholipids was never obtained. Cholesterol recovered from the columns after gel filtration with water or saline was 7 to 30% and 8 to 54% respectively. The amounts of phospholipids remaining on the columns (2 to 8%) were consistently smaller than the corresponding amounts for cholesterol.

*Discussion.* It has been reported that aromatic and heterocyclic compounds have a greater tendency to be adsorbed on the Sephadex gel matrix than other water soluble compounds(12). The present investigation has demonstrated that a more pronounced adsorption of bile acids occurred when the

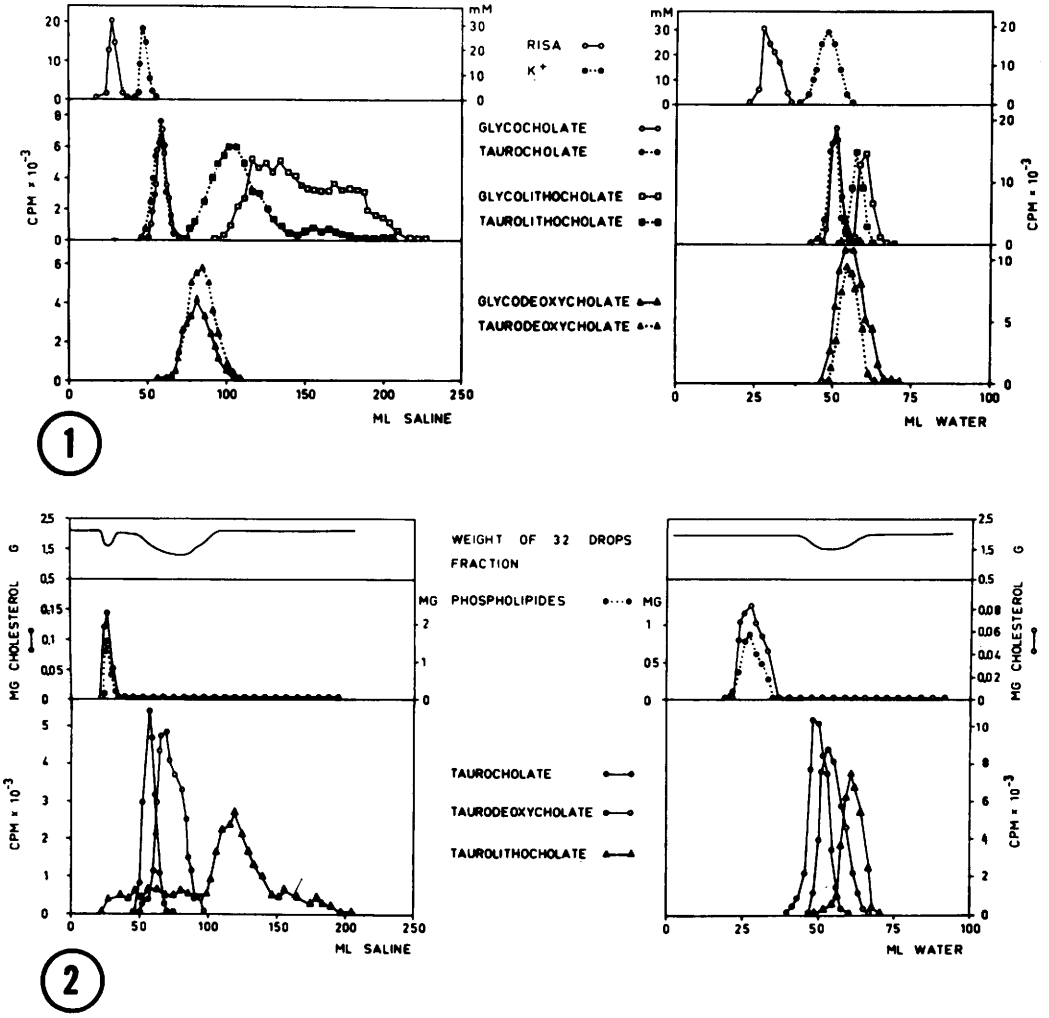


FIG. 1. Gel filtrations of $C^{14}$ labeled conjugated bile acids. Reference: Gel filtration of RISA and potassium chloride.

FIG. 2. Gel filtration of gall bladder bile in which labeled taurine conjugates have been dissolved. Elution curves of surface active compounds (upper part), cholesterol and phospholipids (middle part) and labeled bile acids (lower part) are shown.

gel filtrations were performed with saline than with water. There was a progressive increase in adsorption of conjugates of trihydroxycholanic, dihydroxycholanic and monohydroxycholanic acids. Advantage was taken of this property of Sephadex to achieve separation of these 3 groups of compounds. Furthermore gel filtration might be useful in separation of bile acids from other biliary compounds, such as proteins, phospholipids and cholesterol.

Gel filtration on columns of Sephadex G-25 gives an exclusion of molecules with a molecular weight higher than 3,500-4,500. Gel filtration of bile showed that cholesterol and phospholipids were present in the macromolecular fraction. Similar results of gel filtration of bile on columns of Sephadex G-25 and G-75 have been reported(13). The present investigation has established that the main bile acids in bile, *i.e.* GC, GCD, GD, TC, TCD, and TD are absent from the macromolecular fraction after gel filtration. If there exists in bile a complex between phospholipids and bile acids, as anticipated in the LBS system of Isaksson(2,3), this

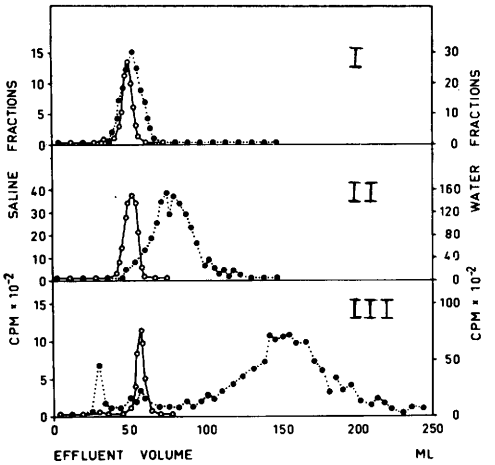


FIG. 3. Gel filtration of gall bladder bile obtained after oral administration of labeled cholic (I), chenodeoxycholic (II) and lithocholic acid (III). Elution curves of labeled metabolites from standard columns eluted with either water (○—○) or saline (●—●).

complex must be easily decomposed by gel filtration. Therefore, supporting evidence for Verschure's theory of the binding of bile acids to the bili-lipoprotein was not obtained. However, compounds containing cholesterol and phospholipids which were isolated in the effluent fractions after gel filtration may only be fragments of the naturally occurring complexes. Investigations on the physical state of cholesterol and phospholipids before and after gel filtration are in progress and preliminary results indicate that there are considerable differences, indicating that the method of gel filtration used caused a decomposition of naturally occurring complexes.

The fact that variable amounts of cholesterol were recovered from the columns is probably explained by a precipitation of cholesterol, which may have occurred when the natural complex was broken during the gel filtration. In view of the degradation of the natural lipid complexes in bile associated with gel filtration, the present application of this technic appears to be of limited value for studying the physical state of lipids in bile.

*Summary.* Gel filtration on Sephadex G-25 was applied to conjugated bile acids in water solution or in bile. 1. An adsorption of bile acids occurs during gel filtration. The adsorption was more pronounced when gel filtration was performed with saline than with water. Conjugates of monohydroxycholanic acid were adsorbed to the greatest extent followed by trihydroxycholanic acid and dihydroxycholanic acid. Rates of elution of taurine and glycine conjugates of a bile acid were about the same. 2. Labeled conjugated bile acids dissolved in bile or *in vivo* labeled conjugates, obtained by administration of the corresponding free acid to patients, showed the same behavior on gel filtration. The conjugates of cholic, deoxycholic and chenodeoxycholic acid were all absent from the molecular weight fraction ($M > 3{,}500$-$4{,}500$) and were eluted at about the same rate as the corresponding conjugates gel filtered in the absence of bile. A small amount of the lithocholic acid conjugates in bile were eluted in the macromolecular fraction. 3. Cholesterol and phospholipids in bile were eluted in the macromolecular fraction and thus were separated from the bile acids. A variable amount of cholesterol was recovered from the columns after gel filtration.

The results of gel filtration of bile constituents are discussed in relation to our present understanding of the physical state of lipids and bile acids in bile.

1. Norman, A., *Proceedings of NATO Advanced Study Institute on the Biliary System*, 1964, in press.

2. Isaksson, B., *Acta Soc. Med. Upsaliensis*, 1953-1954, v59, 296.

3. ———, On the lipides and bile acids in normal and pathological bladder bile, 1954, Diss., Gothenburg.

4. Verschure, J. C. M., *Clin. Chem. Acta*, 1956, v1, 38.

5. Dessi, P., Franco, T., *Boll. Soc. Ital. Biol. Sperimento.*, 1955, v32, 22.

6. Norman, A., PROC. SOC. EXP. BIOL. AND MED., 1964, v775, 936.

7. ———, *Arkiv Kemi*, 1955, v8, 331.

8. Flodin, P., Dextran gels and their application in gel filtration, 1962, Diss., Uppsala.

9. Beling, C. G., *Acta Endocr.*, Suppl. 79, 1963.

10. Hanel, H. K., Dam, H., *Acta Chem. Scand.*, 1955, v9, 677.

11. Chen, P. S., Jr., Toribara, T. Y., Warner, H., *Anal. Chem.*, 1956, v28, 1756.

12. Gelotte, B., *J. Chromatography*, 1960, v3, 330.

13. Tureborn, E., *Nature*, 1963, v197, 1309.

Received February 10, 1964. P.S.E.B.M., 1964, v116.