Electrophoretic Behavior of Bile Acids and Cholesterol in Gall Bladder and Hepatic Bile.* (29083)

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Verschure(1) and Dessi and Franco(2) independently showed that with the aid of paper electrophoresis 4 protein bands could be separated from gall bladder bile. The first band migrating furthest towards the anode, was called bili-lipoprotein(1), the second was identified as albumin(3), the third was a mixture of proteins of alpha and beta mobility(4) and the fourth, which remained at the point of application, was identified as a mucoprotein(5,6).

The bili-lipoprotein is of considerable interest as it contains all of the bilirubin and most of the cholesterol and bile acids present (7,8). Several investigators have shown that protein forms only a small part of the lipoprotein(6) and it has been suggested that it is a mucoprotein(6,9). As only small amounts of the bili-lipoprotein can be isolated from hepatic bile, it has been postulated that the lipoprotein is produced within the gall bladder(1,10). Electrophoresis of hepatic bile has revealed that the bile acids travel with the same mean mobility as albumin and that cholesterol shows no mobility.

The present investigation was concerned with the reported differences in electrophoretic behavior of both cholesterol and bile acids in gall bladder bile and hepatic bile. Because of difficulties in quantitating small amounts of these compounds, previous investigations of this problem had to be conducted on bile samples which had been concentrated several times by dialysis. The use of labeled bile acids and cholesterol made it possible to analyze bile samples containing these components at concentrations within the physiological range.

Experimental. Gall bladder bile was obtained at operation from patients before elective cholecystectomy for cholelithiasis. Hepatic bile was obtained from patients with a T-tube inserted in the common bile duct after choledochotomy for choledocholithiasis. The bile samples were taken after the T-tube had been closed for 3 days and the patients were ambulant and eating the hospital diet *ad libitum*.

The 24-C^{14} labeled free and conjugated bile acids were synthesized and purified as described earlier(11).

Paper electrophoresis was performed in barbiturate buffer of ionic strength 0.1, pH 8.6, in an electrical field of 7.5 V/cm for 3 hours(1). Bile was applied to 4.0 cm wide strips of Whatman No. 1 paper, in the form of a thin band, with a Hamilton syringe. Unless otherwise stated 25 μl of bile were applied. After electrophoresis the strips were dried at 100°C for thirty minutes.

Following paper electrophoresis, the isotope on the paper strips was counted, either stepwise or continuously, in a paper scanner using a Frieseke-Hoepfner windowless counter with a 1 cm slit.

For quantitation of the bile lipids on the strips, the papers were cut into 1 cm pieces and the lipids extracted by a modified Folch extraction technique. The extraction was carried out at room temperature with a mixture of chloroform : methanol 2 : 1. The extract was partitioned with an equal volume of saline and the chloroform layer separated. Following evaporation of the solvent the residue was redissolved in chloroform and aliquots used for the determination of cholesterol(12) phospholipides(13).

Results. Bile acids. The electrophoretic mobilities of C^{14} labeled cholic, deoxycholic and chenodeoxycholic acid and their corresponding taurine and glycine conjugates were determined. When 1 μg of each acid, as the sodium salt dissolved in 25 μl of water, was applied to the paper strips, the isotope determination after electrophoresis showed

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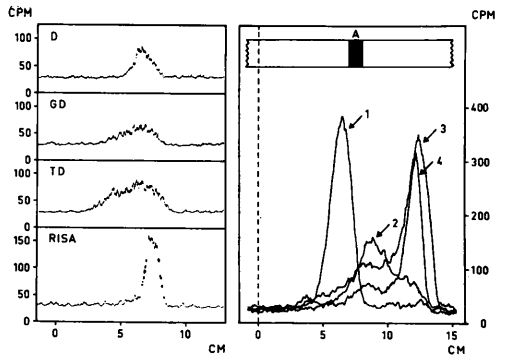

FIG. 1. *Left part:* Paper electrophoresis of labeled deoxycholate (D), glycodeoxycholate (GD) and taurodeoxycholate (TD). Concentration: 0.1%. Reference substance: Radio-iodinated (I^{131}) serum albumin (RISA). *Right part:* Paper electrophoresis of different concentrations of labeled taurodeoxycholate. Concentrations: 0.1 (1), 1 (2), 5 (3) and 10 (4) %. Reference substance: Albumin (A), revealed by staining.

broad peaks all with a mean mobility similar to that of albumin or slightly lower. The results of deoxycholic acid and its conjugates are shown in Fig. 1, left part.

The electrophoretic mobilities of bile acids were influenced by the concentration in the solution applied. As seen in Fig. 1, right part, the main part of the labeled bile acids in a 10% solution of taurodeoxycholate migrated as a band ahead of the albumin. In the decreasing concentration range between 10 and 0.1% there were progressively smaller amounts of bile acids in the high mobility fraction. Similar behavior with different electrophoretic mobilities at different concentrations have been observed for all the conjugates of cholic, chenodeoxycholic and deoxycholic acid.

Bile acids in bile. Cholic or chenodeoxycholic acid-24-C^{14} (5 μc) were given orally to patients and the electrophoretic behavior of the *in vivo* labeled conjugates in gall bladder and in hepatic bile were investigated. Representative electrophoretograms are shown in Fig. 2. The main part of the labeled bile acids in gall bladder bile always travelled as narrow bands ahead of albumin. However, the labeled bile acids in hepatic bile had a slower rate of migration than the bile acids in gall bladder bile.

To investigate the cause of the different mobilities of gall bladder bile acids and hepatic bile acids the influence of the concentration of bile constituents was investigated. Electrophoretic analyses were conducted with dilutions of gall bladder bile in saline and solutions of lyophilized hepatic bile in water. As shown in Fig. 2 similar electro-

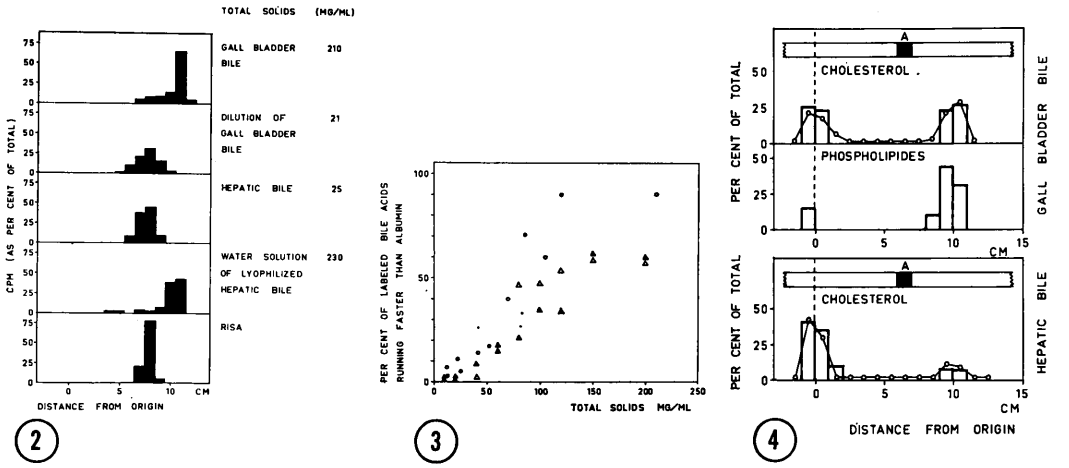

FIG. 2. Paper electrophoresis of bile containing labeled bile acids after oral administration of chenodeoxycholic acid-24-C^{14}.

FIG. 3. Percentage of labeled bile acids with higher mobilities than albumin in dilutions of gall bladder bile (△, ▲) and solutions of lyophilized hepatic bile (○, ●). Bile obtained after oral administration of cholic (△, ○) and chenodeoxycholic acid-24-C^{14} (▲, ●).

FIG. 4. Paper electrophoresis of bile to which trace amounts of cholesterol-C^{14} have been added. Open bars: quantitative determination of cholesterol and phospholides. Circles: labeled cholesterol. Reference substance: Albumin (A), revealed by staining.

TABLE I. Electrophoretic Distribution of Labeled Cholesterol in Solutions of Lyophilized Bile.

Concentration		% of labeled cholesterol in high mobility complex					
Bile solids (mg/ml)	Added tauro-deoxycholate (mg/ml)	Gallbladder bile from patients:			Hepatic bile from patients:		
		L	W	N	H	S	G
200	—	79	38	81	78	73	50
150	—	69	35	80	74	72	34
120	—	70	28	67	67	53	13
100	—	56	34	67	54	50	3
80	—	55	20	56	29	45	7
60	—	39	16	34	36	34	0
40	—	23	6	20	0	14	0
20	—	0	0	0	0	0	0
10	—	0	0	0	0	0	0
200	—	79	38	81		73	50
150	50	100	38	95		100	80
120	80	100	69	100		100	86
100	100	100	87	100		100	100
80	120	100	100	100		100	100
60	140	100	100	100		100	100
40	160	100	100	100		100	100
20	180	100	100	100		100	100
10	190	100	100	100		100	100

phoretic patterns were obtained with diluted gall bladder bile as with hepatic bile of equal total solid concentration. Furthermore, the labeled bile acids in aqueous solution of lyophilized liver bile with a concentration of total solids of 200 mg/ml, migrated as a narrow band ahead of albumin. The percentage of the labeled bile acids travelling ahead of albumin in the electrophoresis of gall bladder bile and lyophilized hepatic bile of different total solid concentrations is shown in Fig. 3. A decrease in concentration lowered the proportion of labeled bile acids with higher electrophoretic mobilities than albumin. Thus the different electrophoretic behavior of gall bladder and hepatic bile acids can be explained by differences in concentrations of bile constituents.

Cholesterol in bile. Because of difficulties in quantitating small amounts of cholesterol with available colorimetric methods cholesterol C^{14} was added to the bile *in vitro* to permit the study of the electrophoretic behavior of cholesterol within the physiological concentrations encountered in bile. A trace amount of cholesterol C^{14} in benzene was added to the test tubes. After evaporating the benzene bile was added. The C^{14} cholesterol was dissolved by mixing with a Vortex homogenizer for 10 minutes.

As shown in Fig. 4 cholesterol in hepatic as well as in gall bladder bile appeared in two forms, one as a high mobility complex traveling ahead of albumin and one not traveling in an electric field. In hepatic bile most of the cholesterol remained at the point of application, whereas in gall bladder bile the major part traveled in the high mobility complex. The Figure also shows that the ratio of labeled to unlabeled cholesterol was the same in the different electrophoretic fractions. This was common to all the bile samples analyzed, thus showing that uniform mixing of the labeled and unlabeled cholesterol was easily obtained. The phospholipides in gall bladder bile were recovered mainly as a high mobility fraction ahead of the albumin. The concentration of phospholipides in hepatic bile was too low to allow quantitation with the methods used.

The influence of the concentration of bile constituents on the ratio of cholesterol in high mobility and immobile fractions has been investigated. In Table I is shown the percentage of labeled cholesterol in the high mobility complex, after electrophoresis of solutions of lyophilized gall bladder and hepatic bile, with different concentrations of total solids. In samples with concentrations of total solids between 120-200 mg/ml varying amounts of cholesterol were present in the high mobility fraction. The percentage of

cholesterol in this complex was decreased by lowering the concentration. Below a concentration of 40 mg/ml almost no cholesterol was present in this fraction. There were considerable differences in distribution of cholesterol in electrophoretograms from different bile samples with equal concentration of total solids. As no differences in principle exist between the electrophoretic behavior of cholesterol between dilute solutions of gall bladder bile and hepatic bile, the different electrophoretic behavior of cholesterol in original gall bladder and hepatic bile can be explained by differences in concentrations of bile constituents.

In a series of experiments, solutions of lyophilized bile were diluted with aqueous solutions of the sodium salts of conjugated bile acids of equal concentrations. Electrophoresis of the different solutions showed that the percentage of labeled cholesterol in the high mobility complex was not reduced by these dilutions. In Table I is shown the experiments with taurodeoxycholate. By decreasing the ratio of cholesterol/taurodeoxycholate, the percentage of cholesterol in high mobility complex was increased.

Methanol extracts of gall bladder bile. Bile was mixed with 50 volumes of methanol and refluxed for 3 hours. The precipitated proteins were filtered off and the extracts evaporated. The residue was dissolved in water to give a final volume equal to that of the original bile. The main part of solids entered into the solution but the solutions were always turbid. Electrophoresis of the redissolved water solutions of the methanol extracts of gall bladder bile with added cholesterol C^{14} or with *in vivo* labeled bile acids was performed. The electrophoretic pictures were about the same as for original bile, *i.e.,* the main part of cholesterol, phospholipides and bile acids moved in the high mobility fractions ahead of the albumin fraction.

Discussion. The results obtained on the mobility of cholesterol and bile acids in gall bladder bile and hepatic bile are in accordance with earlier published results obtained by means of various staining methods(1). Evidence against the hypothesis of a lipoprotein excreted by the gall bladder(1) as an explanation for the different mobilities of cholesterol and bile acids in gall bladder and hepatic bile has been presented. Thus no differences in the mobilities of the two substances in water solution of lyophilized hepatic bile or in gall bladder bile of equal concentration have been obtained. Furthermore no differences in electrophoretic mobilities were observed after electrophoretic analyses of water solutions of methanol extracts of gall bladder bile or gall bladder bile of equal concentration.

It has been shown in the present investigation that the electrophoretic mobilities of conjugated bile acids are dependent on the concentration of the solution applied to the paper strips. This behavior can certainly be explained by the micelle formation of bile salts above the critical concentration. The charges of the bile salt micelles are different from those of single ions(14,15). Concentration differences of bile acids in hepatic and gall bladder bile *per se* can explain the different electrophoretic patterns of bile acids from gall bladder and hepatic bile.

Cholesterol in gall bladder bile must be kept in water solution as a complex. This complex, which is not a lipoprotein, on dilution easily dissociates leaving cholesterol in a form which is not mobile in an electric field. This property is in accordance with the theory that cholesterol in bile is solubilized by micelles of association colloids. Proofs for the theory have earlier been presented by Isaksson(16,17) who has demonstrated that bile acids and lecithin together can solubilize cholesterol in amounts present in bile. The composition of the cholesterol containing high mobility complex is unknown. Electrophoretic analyses of gall bladder bile revealed that both bile acids and phospholipides travel at the same rate as cholesterol in gall bladder bile. These facts, however, cannot be used as a proof that the complex consists of these 3 compounds. The observation that no dissociation of the cholesterol complex appears on dilution of the bile with bile salt solutions indicates that there exists a relation between the dissociation of the high mobility

complex and the lowering of the bile salt concentration. If there exists in bile a combination between bile acids and cholesterol this complex can easily be broken. The relation of phospholipides to this complex has not been studied due to lack of suitable analytical methods.

Several investigators have correlated the percentage of cholesterol in the high mobility complex with different diseases in the biliary system. Before the electrophoretic analyses, the bile samples had to be concentrated by dialysis to enable a colorimetric determination of cholesterol extracted from the paper strips. As the percentage of cholesterol in the high mobility complex is dependent on the concentration of the bile constituents an interpretation of the results is difficult as standardized concentration and electrophoretic techniques were not used. It must be further pointed out that a dissociation of the complex occurs on electrophoresis, by the dilution of the traveling band with the buffer present in the paper. If different amounts of bile are applied to the origin, varying degrees of dilution arise and this results in varying ratios of cholesterol on the line of application and in the high mobility complex.

Summary. 1. Human gall bladder and hepatic bile, obtained after oral administration of cholic and chenodeoxycholic acid-24-C^{14}, were analyzed by paper electrophoresis. Similar studies were performed with bile supplemented *in vitro* with cholesterol C^{14}. 2. In gall bladder bile the main part of the labeled cholesterol and the bile acids migrated faster than albumin. 3. In hepatic bile the bile acids migrated at a rate similar to that of albumin whereas most of the cholesterol remained at the point of application. 4. The differences in electrophoretic mobilities of cholesterol and bile acids in hepatic bile and gall bladder bile respectively can be explained by differences in concentration of the bile constituents. 5. Cholesterol in gall bladder bile is in solution as a complex with a high electrophoretic mobility. This complex is not a lipoprotein and easily dissociates by dilution of the bile into a complex with no electrophoretic mobility. The bile acids are of importance for the formation and stabilization of the high mobility complex.

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