Variations in Pigment and Carbohydrate Content of Gallbladder Bile Affect Accurate Quantitation of Total Protein When Using the Fluorescamine Method

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Background: Despite solute dilution and reduced total lipid concentrations, an unexplained increase in protein concentration has been reported to occur in the gallbladder bile of cholesterol gallstone patients. *Methods:* Solutes in gallbladder bile from gallstone-free controls and from four study groups were measured using standard methods. Total proteins were measured using amino acid analysis and a conventional fluorescamine method. *Results:* Bile salts and pigment content were greater in gallstone-free controls than in all other study groups, including morbidly obese gallstone-free subjects. Total biliary protein concentration, as determined by amino acid analysis in the gallstone-free control group was higher than in non-obese gallstone patients with multiple stones and in morbidly obese gallstone-free subjects. Total biliary proteins as measured with fluorescamine, however, did not show intergroup differences. A major problem of the conventional fluorescamine assay is shown to be an artefact arising from the high pigment content of the more concentrated samples. *Conclusions:* Very dilute gallbladder bile samples are often found in the presence of gallstone disease. This also occurs in morbidly obese subjects, even in the absence of gallstones. Although the contribution of protein secretion/absorption by the gallbladder can also be relevant, especially in the presence of morbid obesity, the protein concentration in gallbladder bile, when accurately measured, generally parallels the concentrations of non-absorbed biliary solutes, reflecting the efficiency of fluid absorption. Measurement of biliary proteins by the conventional fluorescamine method is unreliable in clinical studies in which intergroup differences in pigment content are commonly present.

Key words: Amino acid analysis; fluorescamine assay; gallbladder bile; total biliary proteins

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Under physiologic conditions, bile salts are not absorbed by the gallbladder and comprise, by mass, the largest component of total biliary lipids (1–3). The bile salt concentration in normal human gallbladder bile samples, obtained under fasting conditions, averages from 150 to 200 mmol/l, as a result of the well-known fluid absorption capacity of the gallbladder mucosa (4–7). An impairment of the efficient normal mucosal fluid absorption process in the presence of cholesterol gallstone disease can be considered one of the more likely explanations for the common finding of reduced total lipid concentrations in gallbladder bile during fasting in these patients (5–9). In patients with cholesterol gallstone disease, despite reduced total lipid concentrations, the total protein concentration in gallbladder bile has been estimated to be higher than in gallstone-free controls (7, 10). Such estimates are based on reports using the conventional protein fluorescamine assay (11). These findings of a selective increase in total protein concentration in gallstone patients cannot simply be explained by differences in fluid absorption but could represent differences in the effectiveness of unidentified processes associated with protein absorption or secretion, or both. Alternatively, the differences could be artefactual, arising from systematic errors in solute measurement, especially estimation of protein concentration.

The purpose of the present study was to try to reconcile the discrepancies between the higher total protein and the lower total lipid concentrations previously reported in cholesterol gallstone patients with respect to controls.

The findings show that protein concentrations in gallbladder bile, when accurately measured by amino acid analysis, generally parallel the concentrations of non-absorbed biliary solutes, reflecting the efficiency of fluid absorption. A

selective increase in biliary protein concentration with regard to non-absorbed solutes is consistently found only in morbidly obese gallstone patients and only occasionally in some non-obese gallstone-free and morbidly obese gallstone-free subjects.

PATIENTS AND METHODS

Subjects and sampling of bile

With the approval of the Cleveland Clinic Foundation's Research Projects and Institutional Review Committee Regarding Human Studies, gallbladder bile was obtained by aspirating the gallbladder during surgery after the patients had fasted overnight.

The following subject groups were included: A) 26 non-obese gallstone-free subjects during intra-abdominal surgery for gastric ulcer ($n = 3$), duodenal ulcer ($n = 2$), esophageal reflux ($n = 2$), gallbladder adenomyoma ($n = 1$), gastric carcinoma ($n = 7$), early gastric cancer ($n = 4$), colon cancer ($n = 5$), or small hepatocellular carcinoma in the absence of chronic liver disease ($n = 2$). In the present paper, this heterogeneous control group will be operationally referred to for comparison purposes as the 'gallstone-free control group'; no chemotherapy was administered before surgery in any of the patients; B) 13 non-obese patients with multiple symptomatic cholesterol gallstones found at laparoscopic cholecystectomy; C) 9 non-obese patients with symptomatic solitary cholesterol gallstones found at laparoscopic cholecystectomy; D) 10 morbidly obese subjects with no previous history of biliary disease found to have cholesterol gallstones (9 multiple and 1 solitary) by intraoperative ultrasound at surgery for gastric bypass; E) 20 morbidly obese gallstone-free patients at surgery for gastric bypass.

In patients without gallstones, bile was aspirated after the needle was passed through the anterior lip of the liver and inserted into the gallbladder. In the gallstone groups, bile was sampled immediately after the cystic duct was clamped. Care was taken to reduce stratification of bile before aspiration (12). No significant difference was present in the mean aspirated volume among groups ($P = 0.63$). Absence of gallstones in the stone-free groups was confirmed by either intraoperative ultrasonography or gallbladder palpation. Gallstones were classified as cholesterol in type on the basis of conventional morphologic criteria (5) and by the presence of cholesterol crystals in the sediment after centrifugation of fresh bile (15,000 *g* for 30 min).

Samples were excluded if they showed a positive test for blood contamination (Hemoccult, Smith Kline Diagnostics, Inc., San Jose, Calif., USA) or if evidence of non-neoplastic liver disease, cholestasis, cystic duct obstruction, or biliary sepsis was present. A total lipid concentration of less than 5 g/dl and/or non-opacification of the gallbladder at oral cholecystography has been used as exclusion criteria in previous studies (5–9). These have been selected as exclusion criteria because a non-opacifying gallbladder on oral cholecystography and a very low total lipid concentration in gallbladder bile coexist when the cystic duct is obstructed (13). We decided not to apply these exclusion criteria because we found in the present study that 25% of our morbidly obese gallstone-free subjects had a total lipid concentration of less than 5 g/dl.

Bile samples were carefully mixed, divided into aliquots, immediately frozen at −80°C, and protected from light exposure until analyzed. Every assay run was performed in such a manner that an equal proportion of randomly selected samples from each study group was represented. Histologic examination of the removed gallbladders failed to provide evidence of either acute or marked chronic cholecystitis on the basis of conventional histologic criteria.

Lipid analysis

Bile was thawed, vortexed thoroughly, and extracted in methanol (1:100 v/v) for bile salt and in isopropanol (1:30 v/v) for cholesterol and phospholipid measurements. Extracted samples were centrifuged (3000 *g* for 10 min), and the supernatant was assayed. Total bile salt concentration was measured by the 3α-hydroxysteroid dehydrogenase method (14). Phospholipid concentrations were measured with a kit modification that uses the enzymatic method of Takayama (Pl-Kit K, Nippon Shoji Kaisha, Ltd, Osaka, Japan) (15, 16). Cholesterol concentrations were assayed enzymatically, using a commercially available assay kit (Boehringer-Mannheim Corp., Indianapolis, Ind., USA) (17). Every assay was performed in triplicate, and each also included a sample blank to correct for possible artefact arising from differences in pigment content among samples.

Pigment content

Pigment content was estimated in duplicate as follows: bile was thawed, vortexed thoroughly, and extracted 1:100 (v/v) in methanol. After centrifugation (2000 *g* for 5 min), the supernatant was isolated, and absorption at 450 nm was determined spectrophotometrically.

Total protein concentrations

Amino acid analysis. Amino acid analysis for total biliary protein measurement is conventionally performed after trichloroacetic acid (TCA) precipitation (18). In preliminary studies to determine accuracy in measuring total proteins in concentrated gallbladder bile, we have shown that TCA precipitation causes losses of biliary proteins of about 25% (19). For this reason, we decided to use automated hydrolysis and amino acid analysis of bile without TCA pretreatment as a reference standard for protein determination. Bile was thawed and centrifuged (15,000 *g* for 30 min), and the supernatant was diluted with high-performance liquid chromatography (HPLC) water (dilution range, 1:180 to 1:1000). Norleucine (250 pmol) was added as an internal standard to 40-µl aliquots of the diluted sample, which were then hydrolyzed, derivatized with phenylthiocyanate, and loaded on a PTC C-18

Table I. Demographic characteristics of 78 patients from whom gallbladder bile was aspirated

Characteristic	Non-obese			Morbidly obese	
	Gallstone-free ($n = 26$), mean ± s	Multiple gallstones ($n = 13$), mean ± s	Solitary gallstones ($n = 9$), mean ± s	Gallstone-free ($n = 20$), mean ± s	Gallstones ($n = 10$), mean ± s
Age (years)	51.4 ± 9.2	51.5 ± 4.7	51.9 ± 12.9	37.9 ± 7.3*	33.4 ± 8.2*
Sex (men/women)	18:8	5:8	5:4	5:15	2:8
Weight (kg)	65.2 ± 9.3	69.2 ± 8.6	67.7 ± 4.3	136.1 ± 28.7§	137.3 ± 12.2§

* $P < 0.005$ versus all non-obese groups.
§ $P < 0.001$ versus all non-obese groups.

column for reverse-phase HPLC. A completely automated system was used (Model 420A Derivatizer-Analyzer System, Applied Biosystems, Inc., Foster City, Calif., USA). The hydrolysis conditions were 6N HCl, under argon, and a 1-h vapor phase at 160°C; these conditions are comparable to liquid-phase hydrolysis at 110°C for approximately 30 h. An hydrolysis test peptide (Applied Biosystems, Inc.) with 250 pmol of norleucine was used as a control for the hydrolysis and derivatization procedures. The concentration of each amino acid was determined by automated calculation of the peak area recorded at 254 nm. The instrument was calibrated using amino acid standard H (cat. no. 20088, Pierce Chemical Co., Rockford, Ill., USA) containing 250 pmol of each amino acid and 250 pmol of norleucine. The calibration mixture was subjected to the same hydrolysis, derivatization, and HPLC procedures as the samples. Total protein concentration was calculated as the sum of all amino acids present in proteins except for glycine, which was not included because it is present in large amounts in the samples as the bile salt amidate. All analyses were performed at least in duplicate. A mean intra-assay coefficient of variation of 3% was determined from measurements in triplicate in some samples ($n = 4$).

Because we did not use protein precipitation, our measurement included free amino acids present in bile. For this reason, free amino acids were independently determined in eight samples (four from the gallstone-free controls and four from the multiple gallstone group). After protein precipitation with 4% sulfosalicylic, free amino acids were determined by amino acid analysis, using a Beckman 6300 amino acid analyzer (Fullerton, Calif., USA). Values of less than 0.1 mg/ml were invariably found. On the basis of such values, the free amino acid content was calculated to be in the range of 2% to 10% of total protein for the gallstone-free normals and 1% to 9% of total protein for the multiple gallstone samples. In addition, more than 80% of the free amino acids (representing less than 10% of the total amino acids) were composed of glycine, taurine, and cysteine. Glycine and taurine were not included in the calculation of protein-derived amino acids. Cysteine represents only a very minor percentage of the protein-derived amino acids. On the basis of these data, we concluded that the possibility of overestimating protein concentration by amino acid analyses because of the presence of free amino acids was negligible.

Conventional fluorescamine assay. This assay was performed using a previously described method (11). To minimize the previously described pigment quenching effect (20), each sample was measured at three dilutions (1:750, 1:1500, and 1:3000 cumulative dilutions of native bile before the fluorimetric readings), each of them in triplicate. Only readings falling within the standard curve were used in the final calculations.

Albumin quantitation by enzyme-linked immunosorbent assay (ELISA)

Human serum albumin and rabbit anti-human albumin antibodies were purchased (Sigma Chemical Co., St. Louis, Mo., USA). Optimal conditions for the ELISA were determined by multistep titration of different reagents in the assay (21). Micro-ELISA plates with flat wells (Corning Glass Works, Corning, N.Y., USA) were coated with human serum albumin, 5 µg/ml, and incubated for 2 h at room temperature. The coated plates were then blocked overnight with blocking buffer consisting of phosphate-buffered saline and Tween (150 mmol/l NaCl, pH 7.4; 0.2% Tween 20). Either human serum albumin (the standard) or bile diluted with blocking buffer (range 1:200 to 1:2000) was added to the wells, followed by rabbit anti-human albumin IgG (the primary antibody), and then incubated for 2 h at 37°C. The concentration range of the standard curve was between 0.015 µg/ml and 2.5 µg/ml. A 1:1000 dilution in blocking buffer of goat anti-rabbit IgG alkaline phosphatase conjugate (the labeled secondary antibody) was then added to the wells, which were incubated for 2 h at 37°C. Absorbance was then read with an automated plate reader (Microplate Autoreader EL311, Biotek Instruments Inc., Windooski, Vt., USA), using a reference wavelength of 405 nm. All samples were assayed in triplicate with intra-assay and interassay mean coefficients of variation of 10% and 20%, respectively.

Carbohydrate concentration

Biliary carbohydrate concentration was measured with a periodic acid-Shiff reagent method that was modified as follows (22). Every sample was analyzed in duplicate in glass

Table II. Comparison of gallbladder biliary lipids and pigment content in 78 patients

Variable	Non-obese			Morbidly obese	
	Gallstone-free ($n = 26$), mean ± s	Multiple gallstones ($n = 13$), mean ± s	Solitary gallstones ($n = 9$), mean ± s	Gallstone-free ($n = 20$), mean ± s	Gallstones ($n = 10$), mean ± s
Cholesterol (mM)	17.06 ± 9.17	15.72 ± 5.24	13.25 ± 8.15	13.74 ± 9.2	10.08 ± 5.65
Phospholipids (mM)	35.48 ± 15.43	32.72 ± 16.81	39.42 ± 18.25	36.71 ± 17.8	23.2 ± 12.3
Bile salts (mM)	187.07 ± 57.01	97.32 ± 49.91**	103.83 ± 57.14*	102.88 ± 55.06**	72.85 ± 58.0**
Total lipids (g/dl)	12.54 ± 3.46	7.87 ± 3.24§	8.6 ± 3.92	8.37 ± 4.25§	5.9 ± 3.76**
Pigment content (453 nm optical density)	1.864 ± 0.91	0.914 ± 0.52*†	1.43 ± 0.70‡	0.713 ± 0.38**	0.808 ± 0.53*

* $P < 0.005$; § $P < 0.001$; **$P < 0.0005$, in comparison with gallstone-free normals.
† Measurements available in 12 samples.
‡ Measurements available in 7 samples.

tubes, as was a sample blank. The same supernatant of the centrifuged bile used for the protein assay was diluted with distilled water (range, 1:5 to 1:40) so that the final reading fell within the standard curve (5 to 50 μg/tube). Aliquots (25 μl) of either porcine gastric mucin type III (Sigma Chemical Co.) as a standard or diluted bile were added to every tube and brought to 2 ml with distilled water. The original protocol was then followed. Sample blank values were subtracted from the sample assay values to minimize overestimation resulting from bilirubin absorbance at the assay wavelength of 555 nm.

Statistical analysis

Intergroup differences were analyzed first with the Kruskal–Wallis test. The variables found to differ significantly between groups by this analysis were then submitted to multiple pairwise comparisons using the Wilcoxon rank-sum test. To control for overall error, Bonferroni's correction was used to adjust the alpha level to 0.005 for intergroup differences found significant by the Wilcoxon test. Regression lines were fitted to the data by means of simple linear regression, and the regression lines were compared using the F-test (23). P values of less than 0.05 were considered significant for the regression equations of total proteins as determined by fluorescamine and amino acid analysis (24). Calculations were performed using the SAS software package (SAS Institute, Inc., Cary, N.C., USA).

RESULTS

Patient demographics

Patient demographics are shown in Table I. The morbidly obese patients with and without gallstones were younger than the subjects in the other study groups. The gallstone-free control group had relatively more men than the other study groups. No difference was found in any of the lipid and protein values when the gallstone-free control group was divided in two subgroups on the basis of sex or diagnosis—that is, non-cancer or cancer (data not shown).

Lipid and pigment concentrations

A comparison of bile salt concentrations and pigment content (means ± standard deviation (s) in samples from the patient study groups is shown in Table II. The gallstone-free control group had a significantly higher bile salt concentration that was nearly twofold greater than that of all other groups (Fig. 1). The concentrations of cholesterol and phospholipids did not differ significantly between groups. The gallstone-free control group had a significantly higher total lipid concentration and pigment content than all the other study groups except for the non-obese solitary gallstone group (Table II, Fig. 2).

Total biliary proteins

As shown in Table III and Fig. 3, the gallstone-free control group had a significantly higher total protein concentration when measured by amino acid analysis than both the non-obese multiple gallstone and the morbidly obese gallstone-free groups. Total protein values for both non-obese solitary gallstone patients and morbidly obese gallstone patients did not differ significantly from those of the gallstone-free control group. In addition, there was a striking contrast between the observed relatively high total protein concentrations and the low levels found for both bile salt concentration and pigment content in the morbidly obese gallstone group (Tables II and III). When total protein values, measured by amino acid analysis, were expressed as a ratio of protein to bile salt concentrations, no intergroup difference was detected (Table III). Furthermore, samples with a total lipid concentration lower than 5 g/dl tended to have a protein (measured by amino acid analysis) to bile salt ratio higher (14.90 ± 17.49; $n = 16$) than samples with a total lipid concentration greater than 5 g/dl (5.58 ± 6.45; $n = 62$), but the difference was not statistically significant.

In previous studies that used the conventional fluorescamine method to measure total proteins, the total protein concentration in gallstone-free controls was reduced (7, 10) or similar to (18) that of cholesterol gallstone patients. To reconcile our data with those found in these studies, we

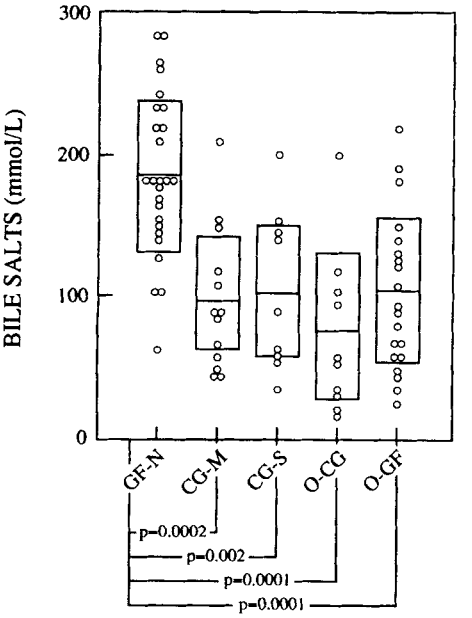

Fig. 1. Bile salt concentrations (including means ± standard deviation) in bile samples from gallstone-free normal subjects (GF-N) compared with those from the four disease study groups: patients with multiple gallstones (CG-M) or with solitary stones (CG-S), morbidly obese subjects with gallstones (O-CG), and morbidly obese subjects without gallstones (O-GF). The bile samples from the gallstone-free normal group had significantly higher bile salt concentrations than those from all other groups.

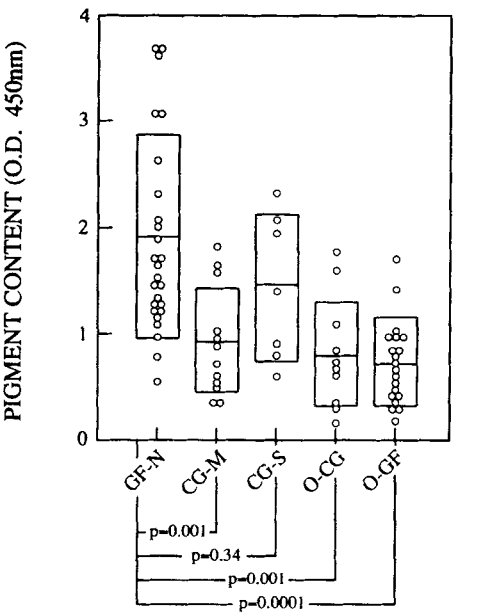

Fig. 2. Pigment content (including means ± standard deviation) in bile samples from gallstone-free normal subjects (GF-N) compared with those from the four disease study groups: patients with multiple gallstones (CG-M) or with solitary stones (CG-S), morbidly obese subjects with gallstones (O-CG), and morbidly obese subjects without gallstones (O-GF). For sample measurement details, see Methods. The bile samples from gallstone-free normals had significantly higher pigment content than those from patients with multiple gallstones and morbidly obese subjects either with or without gallstones.

compared protein estimates from the same samples in the three non-obese groups, using amino acid analysis and the conventional fluorescamine assay.

The intergroup differences found with amino acid analysis were no longer discernible when the fluorescamine method was used (Table III). The percentage of the amino acid value that was represented by the fluorescamine measurement, as an index of discrepancy between the two methods, has been separately plotted against the carbohydrate concentration and the pigment content. A negative correlation ($y = 65.31 + 12.74x$; $r = -0.72$, $P < 0.02$) with the pigment content was found only when samples with optical density (OD) readings greater than 2.0 were considered ($n = 11$). Similarly, a negative correlation ($y = 48.14 + 2.59x$; $r = -0.69$, $P < 0.05$) with the pigment content was found only when samples with carbohydrate concentrations greater than 5 mg/ml were considered ($n = 9$).

Fig. 4A shows a correlation analysis between total biliary protein concentrations obtained with the two different methods using the same samples. Although amino acid analysis and conventional fluorescamine values correlated with each other, the correlation coefficient was higher in the combined multiple and solitary gallstone patients ($y = 0.63 + 1.92x$; $r = 0.95$, $P < 0.0001$) than in the gallstone-free control group ($y = -1.4 + 5.99x$; $r = 0.71$, $P < 0.0001$). This difference resulted from a greater number of outlying values in the gallstone-free control group. Moreover, the two regression lines differed significantly ($P = 0.02$) when analyzed by the F-test. To better compare values in the more commonly encountered range of protein values, the regression lines were calculated and compared after excluding the more extreme amino acid analysis-derived protein values (Fig. 4B). With this modification, correlations between the two assays were high for both groups of patients ($r > 0.90$), and the significance level for the difference between the two lines increased greatly ($P < 0.0001$). Results shown in Table III and Fig. 4 indicate that total biliary protein measurements by the conventional (non-hydrolyzed) fluorescamine method were consistently much lower than those obtained by amino acid analysis. Apart from the correlations, it should also be noted that the protein values obtained by the amino acid analysis method were consistently higher in the gallstone-free control group, which also had a significantly higher pigment content than the non-obese combined gallstone group (Fig. 2).

In separate experiments using 14 samples (7 from the gallstone-free controls, 4 from the non-obese multiple, and 3 from the non-obese solitary gallstone group) we examined the effect of organic solvent delipidation (25) on recovery of total proteins as determined by amino acid analysis (in duplicate in each sample with or without previous delipidation). We found major and variable losses of biliary proteins with delipidation

Table III. Comparison of biliary proteins, albumin, and carbohydrates in gallbladder bile from 78 patients

Variable	Non-obese			Morbidly obese	
	Gallstone-free ($n = 26$), mean ± s	Multiple gallstones ($n = 13$), mean ± s	Solitary gallstones ($n = 9$), mean ± s	Gallstone-free ($n = 20$), mean ± s	Gallstones ($n = 10$), mean ± s
Total proteins amino acid analysis (mg/ml)	5.5 ± 6.84	1.93 ± 1.03*	3.11 ± 1.36	1.73 ± 0.77§	5.32 ± 5.99
Amino acids/bile salts (%)	6.89 ± 9.40	4.56 ± 2.43	7.47 ± 4.79	4.23 ± 2.71	19.44 ± 20.74
Total proteins fluorescamine assay (mg/ml)	1.15 ± 0.81	0.72 ± 0.45	1.23 ± 0.74		
Albumin (μg/ml)	965 ± 1502	181 ± 94	472 ± 286	226 ± 135	893 ± 1560
Carbohydrates (mg/ml)	4.54 ± 3.47	2.95 ± 1.54	2.55 ± 1.11	4.62 ± 2.79	6.11 ± 3.29
Carbohydrates/bile salts (%)	5.53 ± 5.24**	5.37 ± 3.22**	5.56 ± 5.14**	10.13 ± 4.58	24.05 ± 14.59

* $P < 0.005$, in comparison with the non-obese gallstone-free group.
§ $P < 0.001$, in comparison with the non-obese gallstone-free group.
** $P < 0.005$, in comparison with the morbidly obese gallstone group.

ranging from 10% to 60% (mean ± standard deviation, 30.2 ± 21.9). No difference between groups was found.

To investigate the possibility that pigment concentration could interfere with total protein measurements, an additional study (Fig. 5) was performed to compare the accuracy of the two methods (that is, amino acid analysis and fluorescamine) using albumin addition 'spiking' experiments. Bovine serum albumin (BSA) was added in increasing amounts to two bile samples characterized by a major difference in pigment content, one with a high and the other with a low level of pigment. Both had similar bile salt, protein (as assessed by amino acid analysis), and carbohydrate concentrations. For the fluorescamine method the two samples were delipidated (25) before spiking with increasing amounts of BSA. For the amino acid analysis measurements, delipidation was not used. All the spiked samples were appropriately diluted and analyzed in triplicate for each method. Both methods showed a linear increase in total protein estimates (Fig. 5). With amino acid analysis, albumin recovery in the two types of samples was complete, as indicated by the nearly identical slopes (y = 4.99 + 0.93x and y = 3.99 + 0.92x) shown in Fig. 5A.

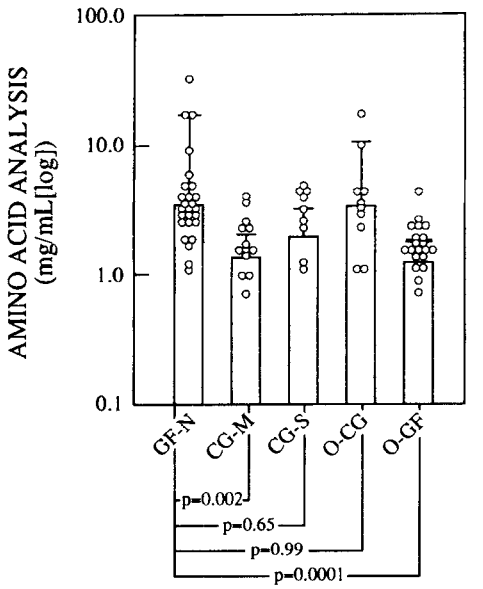

Fig. 3. Total protein concentrations by amino acid analysis (note logarithmic scale) (including means ± standard deviation) in bile samples from gallstone-free normal subjects (GF-N), non-obese patients with multiple gallstones (CG-M) or with solitary stones (CG-S), morbidly obese subjects with gallstones (O-CG), and morbidly obese subjects without gallstones (O-GF). The bile samples from gallstone-free normals had significantly higher total proteins than those from patients with multiple gallstones and morbidly obese subjects without gallstones.

With the fluorescamine assay, by contrast, two main differences were observed (Fig. 5B). The slopes of the regression lines for the high (y = 0.53 + 1.16x) and low (y = 2.41 + 1.49x) pigment content bile samples are distinctly different, indicating that higher pigment content levels reduced albumin recovery. This finding is emphasized by the fact that recovery at all points for the higher pigment content sample is less than recovery at all points for the lower pigment content sample. In addition, at the lower concentrations of added albumin, where the protein-to-pigment ratio is relatively low, the total proteins measured (endogenous in the sample plus added albumin) are lower than the corresponding values obtained with the use of amino acid analysis (Fig. 5A). The delipidation procedure performed before the addition of BSA in the fluorescamine assay also affects the recovery of endogenous proteins, as shown by the lower total protein estimates obtained before BSA was added in the fluorescamine assay as compared with amino acid analysis.

Albumin concentration

Although albumin concentrations (Table III) did not differ significantly between the study groups, a clear trend was seen that parallels the total protein concentrations measured by amino acid analysis.

Total carbohydrates

Although no significant differences in total carbohydrate

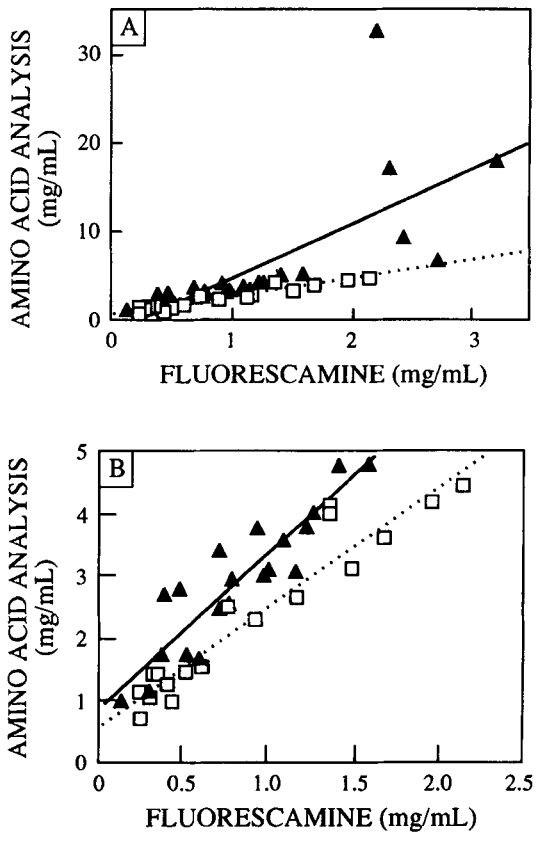

Fig. 4A. Correlation plot for the total protein concentration in the same samples with both amino acid analysis and the conventional fluorescamine method. The same samples from both the gallstone-free normal group (solid triangles and solid line) and non-obese patients with both multiple and solitary gallstones (open squares and interrupted line) were assayed for total protein quantitation by the two methods. A better correlation is found in the gallstone samples ($r = 0.95$) than in the gallstone-free normal group ($r = 0.71$). The two regression lines ($y = 0.63 + 1.92x$ and $y = -1.4 + 5.99x$ for the gallstone group and the gallstone-free normal group, respectively) are significantly different ($P = 0.02$). 4B. The same plot is depicted after exclusion of outlying values. A very high correlation is found in both the gallstone group (interrupted line) ($r = 0.95$) and the gallstone-free normal group (solid line) ($r = 0.91$). The difference between the two regression lines ($y = 0.63 + 1.92x$ and $y = 0.92 + 2.46x$ for the gallstone group and the gallstone-free normal group, respectively) is highly significant ($P < 0.001$).

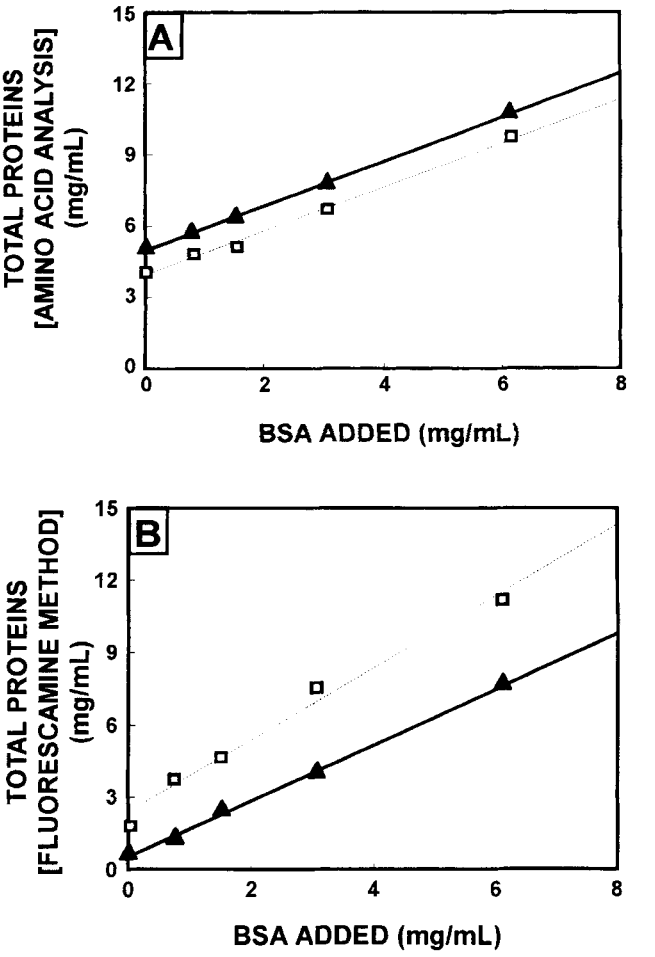

Fig. 5. Comparison of the results of albumin addition ('spiking') experiments at high and low pigment content levels when assayed by amino acid analysis (A) and by the conventional fluorescamine method (B). Albumin (BSA) was added ('spiked') separately in increasing concentrations to two different bile samples, one having a high pigment level (solid triangles and solid line) and the other having a low pigment level (open squares and interrupted line). Each point represents an average of measurements in triplicate. With the fluorescamine assay but not with amino acid analysis, the samples were delipidated before addition of albumin. With amino acid analysis, recovery of albumin from each of the two types of samples is complete, as indicated by the nearly identical slopes ($y = 4.99 + 0.93x$ and $y = 3.99 + 0.92x$) of the two regression lines, as shown in 5A. With the fluorescamine assay, two main differences are observed (5B). The slopes of the two regression lines are distinctly different, and recovery at all points for the higher pigment sample is less than for the lower pigment sample.

concentration were found between the different study groups, a trend toward higher values was seen in the morbidly obese gallstone patient group (Table III). When carbohydrate values were expressed as the ratio of carbohydrate to bile salt concentrations, the morbidly obese gallstone group showed higher values than those found in the gallstone-free controls and in the non-obese multiple and solitary gallstone groups (Table III).

DISCUSSION

Our findings show that total biliary protein concentration as measured by amino acid analysis is significantly higher in the gallstone-free control group than in morbidly obese gallstone-free subjects and in non-obese patients with multiple cholesterol gallstones (Fig. 3). A trend, although not significant, towards reduced total protein concentration compared with gallstone-free controls is also present in non-obese patients with solitary cholesterol gallstones. These findings are consistent with other solute concentration differences (that is, bile salt and pigment) in these groups (Figs. 1 and 2). Neither conjugated bile salts nor conjugated

biliary pigments are effectively absorbed by the gallbladder mucosa (1, 26). Since no patient with pigment gallstones was included in the present study and, hence, no intergroup difference in the rate of bilirubin deconjugation in bile could reasonably be expected, our results suggest that the total protein differences between these groups are primarily due to differences in fluid absorption.

When the protein concentrations were normalized for the different degrees of bile concentration—that is, when protein data were expressed as a ratio to bile salts (Table III)—the intergroup differences found when protein concentrations were expressed in absolute terms disappeared, further proving that, in these groups of patients, fluid absorption is the major determinant of protein concentration. This is particularly true for the non-obese multiple and solitary gallstone patients, in whom the coefficient of variation for protein (Table III) and that for bile salt concentrations and pigment content (Table II) were quite similar. Conversely, the coefficient of variation for protein, on the one hand, and that for bile salt concentrations and pigment content, on the other hand, were widely different in the case of the non-obese gallstone-free controls and of the morbidly obese gallstone-free group. This implies that, in addition to fluid absorption, protein secretion and/or absorption also contributed to the final protein concentration in at least some samples within these groups.

In the present study protein concentrations, as determined by amino acid analysis, were as high in morbidly obese patients with gallstones as in the stone-free control subjects (Fig. 3). Furthermore, the high protein concentration in the morbidly obese gallstone patients was selective and unrelated to other solute concentration differences. This isolated finding of high protein-to-bile salt and protein-to-pigment ratios in morbidly obese, young, asymptomatic gallstone subjects is not the only remarkable and unexplained composition difference that has been found in such patients. A transient marked increase in mucin concentration in the early stages of gallstone disease has also been reported for morbidly obese subjects after gastric bypass surgery (27). These observations are now independently supported by our finding that morbidly obese stone samples tend to have higher carbohydrate content, when expressed as an absolute amount, and contain significantly more carbohydrates when their concentrations are normalized for the different degrees of bile concentration—that is, when carbohydrate data are expressed as a ratio to bile salt concentrations (Table III). The lack of any selective increase in total protein concentrations in our relatively older, symptomatic, non-obese gallstone patients could possibly be explained by a longer elapsed time interval between the sampling of bile and the time by which gallstones eventually formed.

Some protein quantitation studies using the fluorescamine method have shown that the total biliary protein concentration in patients with cholesterol gallstones is either increased (7, 10) or remains about the same (18) compared with gallstone-free controls. With amino acid analysis measurement, however, a relatively small stone-free control group was found to show a trend toward higher values than a larger cholesterol gallstone patient group (18). For these reasons, we compared protein estimates by amino acid analysis and by the conventional fluorescamine assay in the same samples from the three non-obese patient groups. The outcome of these comparisons indicates that protein concentrations determined by the fluorescamine assay are consistently lower than results obtained by amino acid analysis in the same samples. The pattern is especially striking in the control group, leading to the fluorescamine assay being unable to demonstrate the intergroup differences detected by amino acid analysis (Table III). Because posthydrolysis amino acid analysis estimates can reasonably be taken as a reference standard for protein quantitation, these findings indicate that total biliary protein measurements by the conventional (non-hydrolyzed) fluorescamine method yield serious and consistent underestimates of true biliary protein concentrations.

Two factors are primarily responsible for such underestimation. First, TCA precipitation and organic solvent delipidation were both used in the conventional fluorescamine assay but not in the amino acid analysis. Such precipitation and delipidation lead to major protein losses. Losses from TCA precipitation are about 25% (19). We also found major and variable losses of biliary proteins with delipidation of about 30%. Second, like other protein measurement methods, the fluorescamine method has been shown to be susceptible to interference from various substances such as pigments (20). In the present study, the discrepancy between protein measurements obtained by the fluorescamine method and amino acid analysis has been found to negatively correlate with pigment content and with the carbohydrate concentration only when samples with a pigment content greater than 2 OD units or with a carbohydrate concentration greater than 5 mg/ml were considered. These data indirectly prove that either pigments or carbohydrates, above a certain concentration, interfere with the fluorescamine assay. However, the results shown in Fig. 4 and the albumin recovery experiments in Fig. 5 strongly indicate that a major problem of the conventional fluorescamine assay resides in the quenching effect arising from the high pigment content of the more concentrated samples. In particular, after excluding the more extreme amino acid analysis-derived protein values (Fig. 4B), a highly significant difference between the regression line obtained with the fluorescamine method and that obtained with amino acid analysis was found, even though only one of the considered samples had a carbohydrate concentration greater than 5 mg/ml. Furthermore, the possibility that carbohydrates, in addition to pigments, could have accounted for the quenching effect on the fluorescamine method in the BSA-spiking experiments (Fig. 5) is negated by the fact that the two samples chosen had very similar, relatively low carbohydrate concentrations (2.03 and 2.46 mg/ml for the high- and the low-pigmented samples, respectively).

The potential importance of the quenching factor in any

clinical study like this is underscored by the finding that the pigment content of the gallbladder bile is higher in gallstone-free controls than in any of the other study groups. On the basis of all of the above considerations, we conclude that the conventional fluorescamine method is unreliable in clinical studies when intergroup differences in pigment content are likely to be present.

A further factor that could have at least contributed to the relatively high protein concentrations found in gallstone disease in previous papers could be the presence of a bias in patient selection. In fact, an exclusion criterion of a total lipid concentration of less than 5 g/dl and/or of non-opacification of the gallbladder at oral cholecystography has always previously been applied in clinical studies involving gallbladder bile samples (5–9). This exclusion criterion has been instituted on the assumption that a poorly functioning mucosa must always be a consequence of the presence of the stones resulting in cystic duct obstruction and/or chronic inflammatory gallbladder sclerosis with atrophy (13). If this assumption were shown to be invalid, however, highly diluted samples obtained from gallbladders with poor function independent of the presence of the stones would have been excluded. This, in turn, would in previous studies have contributed to a systematic overestimation of total protein concentrations in gallstone disease. Our present findings strongly support the latter probability, for two reasons. First, our data show that severe dilution (less than 5 g/dl) frequently occurs even in the absence of gallstone disease (25% of our morbidly obese gallstone-free patients). In keeping with this, a recent study showed that 34% (11 of 32) of the patients with a non-opacifying gallbladder at oral cholecystography did not have gallstones when examined with ultrasonography (28). Second, a total lipid concentration of less than 5 g/dl can often occur in gallstone disease despite an absence of cystic duct obstruction or fibrotic gallbladder, simply as a result of impaired mucosal fluid absorption. In support of this, 34% (11 of 32) of all our gallstone patients showed a total lipid concentration of less than 5 g/dl. In addition, 29% (23 of 78) of gallstone patients in an epidemiologic survey conducted by the GREPCO (Rome Group for the Epidemiology and Prevention of Cholelithiasis) (29) and 33% (11 of 33) in a clinical study (30) had a non-functioning gallbladder on oral cholecystography despite the absence of clinical and ultrasonographic evidence of cystic duct obstruction or fibrotic gallbladder.

To conclude, the findings of this study clearly indicate that protein concentrations in gallbladder bile, when accurately measured, generally parallel the concentrations of biliary non-absorbed solutes, largely reflecting the efficiency of fluid absorption. A selective increase in protein concentration, mostly as glycoproteins, is consistently found only in morbidly obese gallstone patients and only occasionally in some non-obese gallstone-free and morbidly obese gallstone-free subjects.

Measurement of biliary proteins by the conventional fluorescamine method is unreliable in clinical studies when intergroup differences in pigment content and carbohydrate concentrations of gallbladder bile are present. In the absence of cystic duct obstruction or fibrotic gallbladder, the use of a total lipid concentration of less than 5 g/dl as an exclusion criterion in studies involving gallbladder bile samples is no longer necessary.

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