

Biliary proteins: assessment of quantitative techniques and comparison in gallstone and nongallstone subjects

Kiyoshi Yamazaki, Stephen P. Powers, and Nicholas F. LaRusso

Gastroenterology Research Unit, Mayo Medical School, Clinic, and Foundation, Rochester, MN 55905

Abstract Although protein is the third most abundant solid in bile and is important in cholesterol crystal formation, methods for quantitating the concentration of total protein in bile have not been systematically evaluated. To establish a reliable protein assay for bile, we evaluated three protein assays (Lowry's method and the fluorescamine and Coomassie blue methods), and employed amino acid analysis as a reference technique. Large protein-to-protein variations were observed with the fluorescamine and Coomassie blue methods. Although all assays were affected by interfering substances, Lowry's method and the fluorescamine technique (after trichloroacetic acid precipitation and delipidation of bile) and the Coomassie blue method with native bile showed excellent correlations ($P < 0.0001$) with those obtained by amino acid analysis. Using these reliable protein assays, we examined gallbladder bile obtained at surgery from subjects with and without gallstones. No differences in the concentrations of total biliary proteins were observed among patients with cholesterol ($n = 23$) or pigment ($n = 7$) gallstones and subjects without gallstones ($n = 10$). Protein values obtained by amino acid analysis also did not differ among groups. As expected, bile from patients with cholesterol gallstones was supersaturated with cholesterol while bile from nongallstone subjects and those with pigment stones was unsaturated. ■ These results indicate that it is not possible to separate patients with and without gallstones on the basis of the total protein concentration of gallbladder bile. — Yamazaki, K., S. P. Powers, and N. F. LaRusso. Biliary proteins: assessment of quantitative techniques and comparison in gallstone and nongallstone subjects. *J. Lipid Res.* 1988. 29: 1055-1063.

Supplementary key words protein assays • gallstone pathogenesis

Supersaturation of gallbladder bile in cholesterol is the first step in cholesterol gallstone formation (1). While necessary, supersaturation is not sufficient to completely explain gallstone formation since many subjects with supersaturated bile do not have gallstones (2, 3). Recently, substances in bile, which we have termed nucleation modifiers, have been implicated in cholesterol crystal formation (4). Although the mechanism of cholesterol crystal formation and the precise role of nucleation modifiers in this process have not been well characterized, recent evi-

dence suggests that the nucleation modifiers in bile are proteins (5-8). Indeed, proteins are the third most abundant solid in bile (9) and include specific glycoproteins (5) and apolipoproteins (10, 11) that, respectively, can promote (5) or inhibit cholesterol crystal nucleation (7). For these and other reasons, we (10) and others (4) have proposed that quantitative and/or qualitative abnormalities in biliary proteins may be important in the pathogenesis of cholesterol gallstones.

Several groups (8-10, 12, 13), including our own, have reported results of measurements of total protein concentrations in samples of bile obtained from subjects with and without gallstones. Unfortunately, reputable investigators have obtained different results, reporting higher (8, 13), lower (10), and similar (9, 12) concentrations of total protein in the bile of subjects with gallstones compared to levels in subjects without stones. While subject and sample heterogeneity may contribute to these different results, differences in the techniques used by these investigators to quantitate total biliary protein concentrations are likely of paramount importance. Bile is quite different from other biological fluids because of its unique color and high lipid concentration, and biliary constituents such as pigments and lipids may affect protein measurement. Indeed, widely varying values for total protein concentration have been reported in normal human gallbladder bile [from 0.2 mg/ml (12) to 31 mg/ml (10)], and even in normal rat bile [from 1.6 mg/ml (14) to 20 mg/ml (15)].

Thus, the aims of this study were to establish a reliable, precise, and accurate method for measuring total protein concentration in bile and to compare the total protein concentrations of gallbladder bile obtained from subjects with and without gallstones.

Abbreviation: TCA, trichloroacetic acid.

MATERIALS AND METHODS

Bile samples

Bile was obtained from male Sprague-Dawley rats (250 g) prepared with bile fistulae as described (16). Special care was taken to collect bile from the common bile duct within 2 mm of the hepatic duct bifurcation to avoid contamination of bile with proteolytic enzymes from the pancreas (17).

Human gallbladder bile was obtained by puncture of the gallbladder at surgery and frozen at -20°C until analysis. Biliary protein concentration did not change over 6 months of storage by each of three methods (see below) of analysis. Samples were obtained from 10 subjects without gallstones undergoing laparotomy for reasons unrelated to hepatobiliary diseases. Samples were also obtained from 23 patients with cholesterol gallstones and from 7 patients with pigment gallstones. Gross appearance (18) was used to judge stone composition. All patients had normal liver tests and functioning gallbladders as judged by the total lipid concentration being over 5 g/dl (19). This study was approved by the Institutional Review Board of the Mayo Clinic.

Protein assays

Three methods for measuring total protein [the method of Lowry et al. (20), the fluorescamine method (21), and a Coomassie blue dye-binding assay (22)] were chosen for systematic evaluation. Each method is based on a different principle.

In the method of Lowry et al., bile was diluted with saline (10- to 15-fold for rat bile and 30- to 100-fold for human gallbladder bile). The sample (200 μl) was incubated with 2.5 ml of alkaline copper reagent (20) for 15 min followed by the addition of 250 μl of phenol reagent (Fisher Scientific Co., Fairlawn, NJ). After 45 min, absorbance was measured at a wavelength of 650 nm on a spectrophotometer (Model 34, Beckman Instruments, Inc., Scientific Instruments Division, Irvine, CA).

In the fluorescamine assay, bile was diluted with deionized water (10- to 100-fold for rat bile and 20- to 100-fold for human gallbladder bile). Deionized water with the resistance of 16 megohm-cm was obtained by Bahnstead nanopure ion exchange system (Bahnstead Co., Boston, MA). After addition of 1 ml borate buffer (pH 9.0) to the sample (500 μl), 500 μl of fluorescamine (Pierce Chemical Co., Rockford, IL) solution (20 mg fluorescamine/100 ml acetone) was added and fluorescence was measured after 15 min at an excitation wavelength of 390 nm and an emission wavelength of 480 nm by fluorescence spectrophotometry (LS-3, Perkin-Elmer Corp., Norwalk, CT).

For the Coomassie blue dye-binding assay, bile was diluted with deionized water (50- to 100-fold for rat bile and 400- to 1600-fold for human gallbladder bile). A com-

mercially available kit (Bio-Rad protein assay, Bio-Rad Laboratories, Richmond, CA) was used. Absorbance was measured by spectrophotometry at a wavelength of 595 nm 15 min after the addition of the Coomassie blue reagent (200 μl) to the bile sample (800 μl).

Protein-to-protein variation was examined by using both bovine serum albumin (Bio-Rad) and bovine serum γ -globulin (Bio-Rad) as standards, since albumin and globulin are likely two of the most abundant proteins in human bile (23–25). Results of bile analysis by gel filtration and high performance liquid chromatography on a Bio-Sil TSK-250 column (Bio-Rad) using protein standards and by polyacrylamide gel electrophoresis (10% polyacrylamide gel) with sodium dodecyl sulfate according to Laemmli (26) confirmed the abundance of albumin and γ -globulin in human gallbladder bile (data not shown).

The effects of interfering substances in bile on protein measurements were determined by comparing results of measurements before and after several separatory procedures including dialysis, precipitation with trichloroacetic acid (TCA), delipidation, or a combination of these maneuvers. Dialysis was performed against 0.01 M Tris-buffer (pH 7.4) for 24 hr using dialysis membrane (Spectra/Por 6, Spectrum Medical Industries, Los Angeles, CA) with a molecular weight cutoff of 2,000. For TCA precipitation, diluted samples were brought up to 1 ml with saline or deionized water and 1 ml of 20% TCA (final concentration 10%) was added, incubated for 30 min at 4°C , and centrifuged at 1700 g for 30 min at 4°C . The pellet was resuspended in 2 ml of 10% TCA and recentrifuged. The final pellet was dissolved in borate buffer (pH 9.0) (200 μl for Lowry's method, 500 μl for fluorescamine method, and 800 μl for Coomassie blue method) and incubated overnight at 4°C to assure complete solubility. For delipidation, the final pellet after TCA precipitation was washed in 3 ml of diethyl ether-ethanol 3:1 (vol/vol) and centrifuged at 1700 g for 30 min at 4°C . This washing procedure was repeated two times and the resultant pellet was dissolved in borate buffer (pH 9.0) and incubated as above. Although concentrated bile samples retain some color after TCA-precipitation and delipidation, background absorbance (for the Lowry and Coomassie blue methods) or fluorescence (for the fluorescamine method) was never greater than 5% of absorbance or fluorescence readings for bile samples yielding protein values within the range of our standard curve.

The accuracy of each of the three methods was assessed by comparing results obtained by each technique with those determined by amino acid analysis. Bile was precipitated with TCA as above and acid-hydrolyzed with 6 N HCl at 110°C for 20 hr. Amino acid composition was determined on an amino acid autoanalyzer (System 7300, Beckman Instruments, Inc., Spinco Division, Palo Alto, CA). The absolute concentration of protein in a sample

was calculated by summing the weights of the individual amino acids employing 10 nmol of β -alanine as internal standard. The weight of glycine was omitted from the calculation since glycine originating from bile acids could potentially affect the glycine concentration even after dialysis and TCA-precipitation. As the glycine content of most of over 200 proteins is less than 10% (27), the effect of omitting glycine from the calculation is considered negligible.

To quantitate the loss of protein that might occur during TCA-precipitation and delipidation, we added three radiolabeled proteins spanning a range of molecular weights [bovine serum albumin, M_r 66,000 (Bio-Rad), human prolactin, M_r 23,000 (Calbiochem, San Diego, CA), porcine glucagon, M_r 3674] to bile and measured their recoveries. We radiolabeled albumin and prolactin with ^{125}I using the chloramine-T method (28) and purchased porcine ^{125}I -labeled glucagon (Cambridge Medical Technology, Inc., Billerica, MA). Recovery after TCA-precipitation and delipidation was examined by dividing the radioactivity remaining at each step of the preparation by the amount of radioactivity added directly to the bile sample (approximately 100,000 cpm).

Lipid measurement

Total bile acids in bile were measured by an automated modification of the method of Talalay (29). Biliary cholesterol was measured by spectrometry using the kit of Boehringer-Mannheim (30). Biliary phospholipids were

measured by the method of Takayama et al. (31) as applied to the analysis of bile by Gurantz, Laker, and Hofmann (32) with the use of a commercial kit (PL-Kit K, Nippon Shoji Kaisha, Ltd., Osaka, Japan). The cholesterol saturation index was calculated using Carey's table (33).

RESULTS

We examined the protein-to-protein variation of the three assays by assessing differences in measurements resulting from using either albumin or globulin as protein standards. As shown in Fig. 1, the two proteins reacted similarly in the method of Lowry et al. (20). In the fluorescamine and Coomassie blue methods, however, use of albumin resulted in a two- to threefold higher reactivity than comparable amounts of globulin.

The effect of TCA-precipitation and delipidation of the standard solution of albumin is shown in Fig. 2. In Lowry's method and the fluorescamine method, pretreatment by TCA-precipitation or delipidation did not affect the standard curve. Conversely, in the Coomassie blue method, a moderate decrease (0.1 to 0.2 absorbance units) of reactivity was observed. The measurable range of protein concentration with native bile was 20–500 $\mu\text{g}/\text{ml}$ by Lowry's method (20), 1–500 $\mu\text{g}/\text{ml}$ by the fluorescamine method (21), and 1–25 $\mu\text{g}/\text{ml}$ by the Coomassie blue method (22).

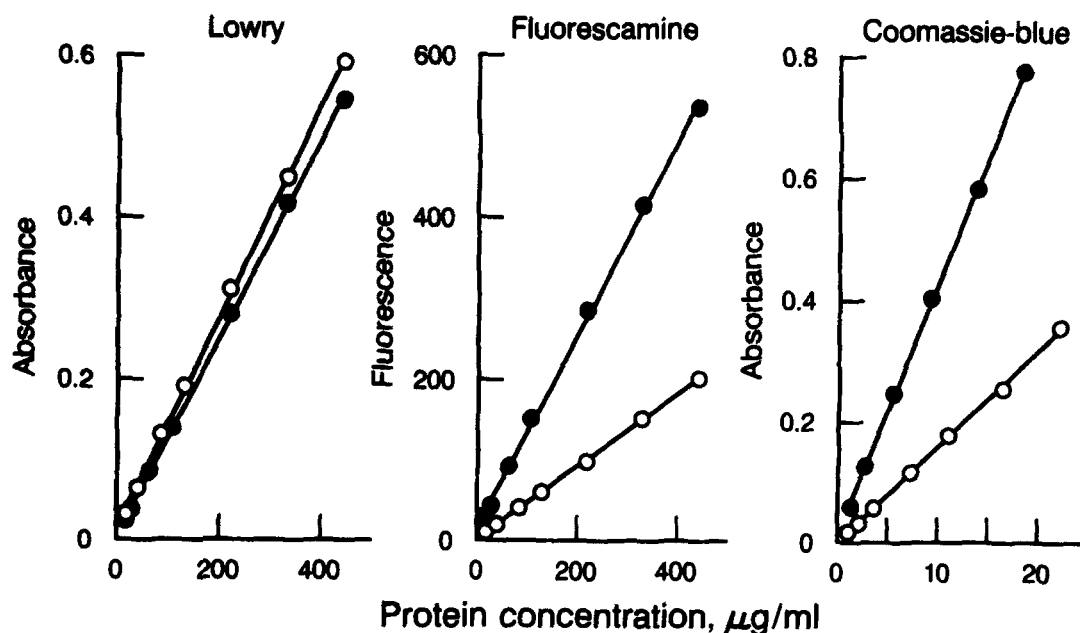


Fig. 1. Protein-to-protein variation between bovine serum albumin (●—●) and bovine serum γ -globulin (○—○) with three protein assays. Data represent the mean of triplicate measurements. For the Lowry method, absorbance was measured at a wavelength of 650 nm. For the fluorescamine method, fluorescence was measured at an excitation wave length of 390 nm and an emission wave length of 480 nm. For the Coomassie blue method, absorbance was measured at a wavelength of 595 nm.

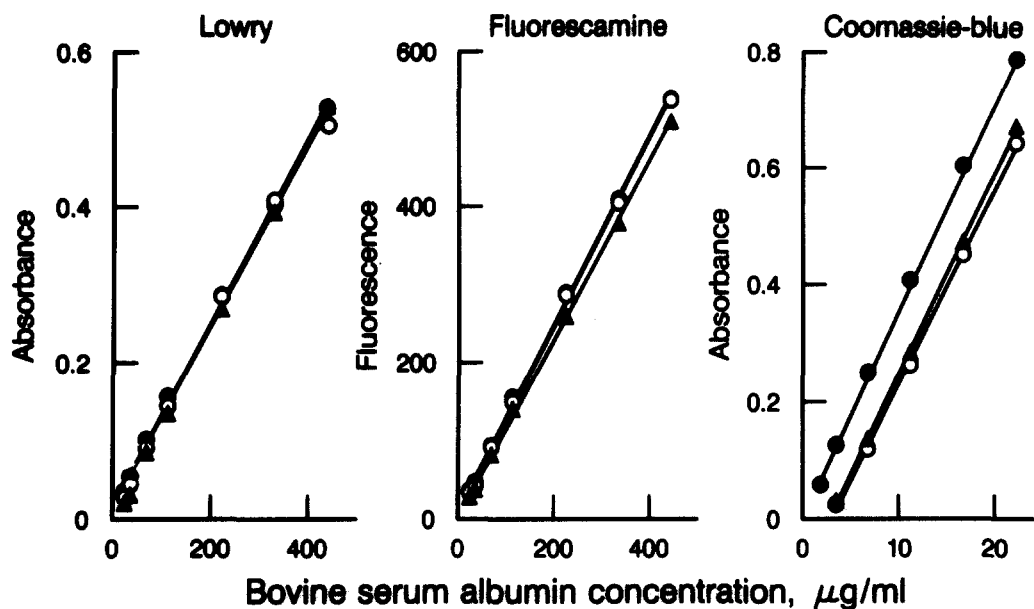


Fig. 2. The effects of TCA-precipitation and delipidation on the standard curve of bovine serum albumin with three protein assays; no pretreatment (●—●), after TCA-precipitation (○—○), and after TCA-precipitation and delipidation (▲—▲). For the Lowry method, absorbance was measured at a wavelength of 650 nm. For the fluorescamine method, fluorescence was measured at an excitation wave length of 390 nm and an emission wave length of 480 nm. For the Coomassie blue method, absorbance was measured at a wavelength of 595 nm.

Total protein concentration in rat bile ($n = 10$) is shown in Table 1. Total protein concentration in native rat bile (i.e., without pretreatment) was 2.62 mg/ml by Lowry's method, 8.48 mg/ml by the fluorescamine method, and 0.75 mg/ml by the Coomassie blue method. After dialysis or TCA-precipitation, significant ($P < 0.0001$) decreases were observed by Lowry's method and by the fluorescamine method. With the Coomassie blue method, a significant decrease ($P < 0.0003$) was apparent after dialysis but not after TCA-precipitation ($P = 0.18$). The lowest values were obtained after TCA-precipitation and delipidation (1.39 mg/ml by Lowry's method, 0.40 mg/ml by the fluorescamine method, and 0.42 mg/ml by the Coomassie blue method). The value for the protein concentration in rat bile after TCA-precipitation obtained by amino acid analysis was 0.53 ± 0.02 mg/ml. As the range of protein concentration obtained by amino acid analysis was very narrow (0.44–0.63 mg/ml), it was difficult to assess the correlation among individual results with the three

methods and those by amino acid analysis. Significant correlations with results from amino acid analysis were found, however, with Lowry's method after TCA-precipitation and delipidation ($r = 0.71$, $P < 0.03$), the fluorescamine method after TCA-precipitation ($r = 0.81$, $P < 0.01$), and the Coomassie blue method without pretreatment ($r = 0.79$, $P < 0.01$).

As shown in Table 2, total protein concentration in 40 gallbladder bile samples also differed largely according to the analytic method used and the type of pretreatment employed. Total protein concentration in native bile was 16.2 ± 1.2 mg/ml by Lowry's method, 5.9 ± 0.6 mg/ml by the fluorescamine method, and 7.4 ± 0.6 mg/ml by the Coomassie blue method. After TCA-precipitation and delipidation, the concentration was 5.1 ± 0.6 mg/ml by Lowry's method, 2.0 ± 0.2 mg/ml by the fluorescamine method, and 3.1 ± 0.3 mg/ml by the Coomassie blue method. The value for the protein concentration in human gallbladder bile after TCA-

TABLE 1. Concentration of total protein in rat bile

	Lowry's Method	Fluorescamine Method	Coomassie Blue Method
	<i>mg/ml</i>		
Native bile	2.62 ± 0.10	8.48 ± 0.20	0.75 ± 0.03
After dialysis	1.66 ± 0.10	1.00 ± 0.09	0.55 ± 0.04
After TCA-precipitation	1.73 ± 0.08	0.45 ± 0.01	0.70 ± 0.02
After TCA-precipitation and delipidation	1.39 ± 0.05	0.40 ± 0.01	0.42 ± 0.04

Data are expressed as mean (\pm SEM) of 10 samples. Value obtained by amino acid analysis after TCA-precipitation was 0.53 ± 0.02 mg/ml.

TABLE 2. Concentration of total protein in human gallbladder bile

	Lowry's Method	Fluorescamine Method	Coomassie Blue Method
	<i>mg/ml</i>		
Native bile	16.2 ± 1.2	5.9 ± 0.6	7.4 ± 0.6
After TCA-precipitation	13.3 ± 0.9	2.3 ± 0.2	7.3 ± 0.4
After TCA-precipitation and delipidation	5.1 ± 0.6	2.0 ± 0.2	3.1 ± 0.3

Data are expressed as mean (± SEM) for 40 samples of bile obtained by puncture of the gallbladder from subjects without gallstones. Value obtained by amino acid analysis after TCA-precipitation was 3.1 ± 0.3 mg/ml.

precipitation obtained by amino acid analysis was 3.1 ± 0.3 mg/ml.

To test the accuracy of each of the methods, 40 samples of human gallbladder bile were subjected to amino acid analysis and correlations were examined between the results obtained by each of the three methods and those obtained by amino acid analysis. The results obtained by any method showed significant correlations with those obtained by amino acid analysis ($r = 0.48$ to 0.85 , $P = 0.002$ to 0.0001). However, the best correlations were obtained by Lowry's method or the fluorescamine method after TCA-precipitation and delipidation ($r = 0.78$, $P = 0.0001$ and $r = 0.76$, $P = 0.0001$, respectively) and the Coomassie blue method with native bile ($r = 0.85$, $P = 0.0001$).

To assess the effect of pretreatment (i.e., TCA-precipitation and delipidation) on protein recovery in each assay, approximately 100,000 cpm of radiolabeled proteins were added to 20-, 100-, 400-, and 800-fold diluted samples of human gallbladder bile. The recoveries of ^{125}I -labeled albumin after the pretreatments were 80–90% in 20- and 100-fold diluted bile (dilution for Lowry's method and fluorescamine method) and 60–75% in 400- and 800-fold diluted bile (dilution for Coomassie blue method). Similar results were obtained with ^{125}I -labeled prolactin and ^{125}I -labeled glucagon (data not shown).

The total protein concentrations in samples of human gallbladder bile obtained from patients without gallstones ($n = 10$), from patients with cholesterol gallstones ($n = 23$), or from those with pigment gallstones ($n = 7$) were investigated using Lowry's method and the fluorescamine assay after TCA-precipitation and delipidation, and the Coomassie blue method without pretreatment (Fig. 3). No statistically significant differences among the three groups were observed by any of the three methods. After TCA-precipitation and delipidation, significant differences were still not apparent with the Coomassie blue method (data not shown). Furthermore, no differences in total protein concentrations among the three groups of subjects were seen by amino acid analysis (Fig. 3). When total protein concentrations in bile were normalized to individual or total lipid concentrations or to cholesterol

saturation (see below), significant differences among the three groups were still not observed.

Table 3 shows the lipid composition and cholesterol saturation index of the samples of human gallbladder bile. Although bile with cholesterol gallstones showed significantly higher ($P < 0.01$) cholesterol saturation index and molar percentage of cholesterol than nonstone bile, no significant differences were found in the molar percentages of bile acids, phospholipids, and total lipid concentrations between cholesterol stone bile and nonstone bile. Compared to pigment stone bile, cholesterol stone bile showed a significantly lower molar percentage of total bile acids ($P < 0.01$) and total lipid concentration ($P < 0.02$), and a significantly higher saturation index ($P < 0.004$).

DISCUSSION

The major findings in this study relate to the reliability and accuracy of different assays for total protein in bile and to possible differences in the total protein concentration of gallbladder bile from subjects with and without gallstones.

Evaluation of protein assays in bile

Although large variations in the concentration of total protein in human bile (8–10, 12, 13) and in rat bile (14, 15) have been reported, and although bile contains pigments, lipids, and free amino acids that can interfere with protein measurements, there have been no reports in which methods for measuring total biliary protein have been systematically evaluated, although a single report has compared results of the fluorescamine method to results obtained by amino acid analysis (8). In contrast, the reliability of protein assays in other biological fluids, including urine (34), cerebrospinal fluid (35) and saliva (36), has been extensively evaluated. These considerations, as well as evolving interest in the possible roles of biliary proteins in the pathogenesis of gallstones, or as markers for hepatic excretory pathways (11, 16) and possible osmotic agents which stimulate bile flow (37), make rigorous assessment

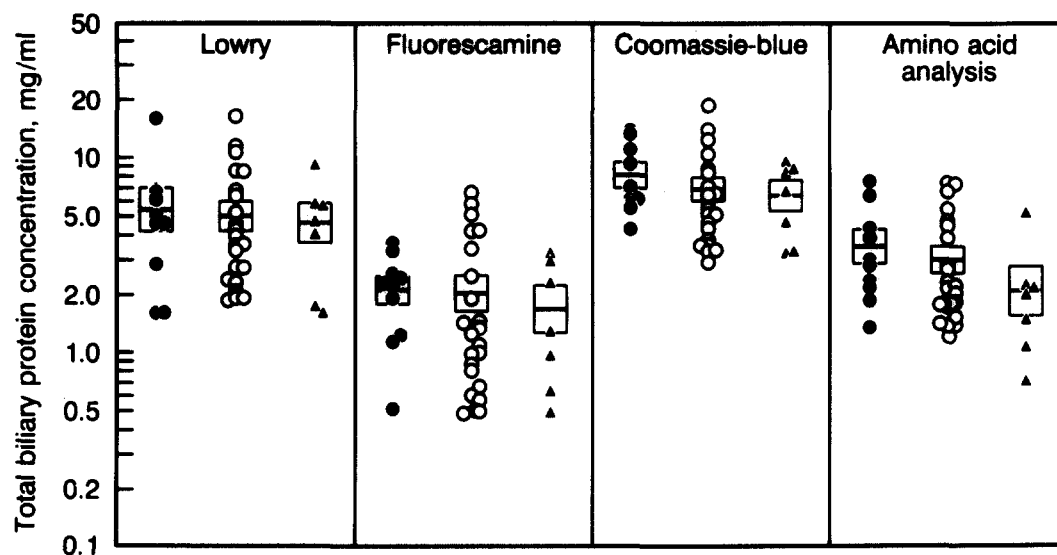


Fig. 3. Comparison of protein concentration in bile among subjects without gallstones (●) and those with cholesterol (○) or pigment (▲) gallstones.

of methods for measuring total protein concentrations in bile necessary and timely.

An ideal assay for total biliary protein concentration should show little effect of the potential interfering substances in bile, display small protein-to-protein variation (i.e., give similar results with different protein standards), and be accurate. Regarding these characteristics, we evaluated three protein assays that are based on different principles. The method of Lowry et al. is a colorimetric assay dependent on the formation of a protein-copper complex and color enhancement by the addition of phenol reagent (20). This method is known to be affected by many interfering substances (38). The fluorescamine assay is a fluorimetric method (21) based on the reaction between fluorescamine and primary amines. While the reaction is considered to be relatively free from interference by non-amine substances, interference by free amino acids is significant (38). Finally, the Coomassie blue method is a colorimetric assay based on the binding of proteins to this dye (22). While the number of interfering

substances is considered to be small (38), and while free amino acids do not interfere with the measurement because the reagent does not react with peptides smaller than nine amino acids (39), pigments such as bilirubin are likely to affect this colorimetric assay. Results by all three methods may vary depending on the standards used (20–22).

Our results assessing protein-to-protein variation suggest that bovine serum albumin is a reasonable protein standard to employ for rodent or human bile. Protein concentrations obtained with albumin correlated well with those obtained by amino acid analysis. Moreover, albumin is a major protein in bile. While our results (Fig. 1) suggest that γ -globulin may also serve as a suitable standard in the Lowry assay for bile, variation with the use of γ -globulin standards will likely occur with the two other methods.

We evaluated several approaches, including dialysis, TCA-precipitation, and TCA-precipitation plus delipidation to remove interfering substances from rodent and

TABLE 3. Lipid composition of nonstone bile and stone bile

	Nonstone Bile (n = 10)	Cholesterol Stone Bile (n = 23)	Pigment Stone Bile (n = 7)
Cholesterol saturation index	0.98 ± 0.07	1.28 ± 0.06 ^a	0.89 ± 0.09
Total lipid concentration (g/dl)	10.1 ± 1.1	9.0 ± 0.6	12.5 ± 1.2 ^b
Bile acid (molar %)	74.4 ± 1.4	71.5 ± 1.1	77.6 ± 2.0 ^c
Phospholipids (molar %)	18.6 ± 0.9	18.8 ± 0.6	16.7 ± 1.5
Cholesterol (molar %)	7.0 ± 0.6	9.7 ± 0.6 ^d	5.8 ± 0.7

^a $P < 0.01$, compared to nonstone bile; $P < 0.004$, compared to pigment stone bile.

^b $P < 0.01$, compared to cholesterol stone bile.

^c $P < 0.01$, compared to cholesterol stone bile.

^d $P < 0.02$, compared to nonstone bile; $P < 0.002$, compared to pigment stone bile.

human bile. These maneuvers resulted in substantial (>50%) decreases in the measured values of biliary protein. The progressive decrease in measurable values for protein after successive preparative steps was almost certainly due to the removal of substances that interfere with the assays; progressive loss of protein is an unlikely alternative explanation given results of our recovery experiments using radiolabeled proteins. Differences in measurable values were most evident with the fluorescamine assay after dialysis of rat and human bile (Tables 1 and 2), suggesting removal primarily of amino acids. A decrease in the measured values of protein was also evident after dialysis using either the Lowry or the Coomassie blue methods, suggesting the removal of interfering substances with molecular weights of less than 2000. The greater decrease occurring with TCA-precipitation plus delipidation, particularly evident with human bile, suggests that interfering substances of higher molecular weight are also being removed. The apparent differences in the effects of these maneuvers on rat and human bile also raise the possibility that there may be different amounts or types of interfering substances in rodent and human bile.

We assessed the accuracy of the three assays for biliary proteins using amino acid analysis. Although this technique is not ideal for routine measurements because it is tedious, time-consuming, and requires expensive equipment, it is considered accurate (38) and has been used as a reference method for protein assays by others (8, 40). When measurements were made on human bile samples using albumin as standard, all three methods gave results that had significant correlations with those obtained by amino acid analysis; the best correlation was found with Lowry's method, the fluorescamine assay after TCA-precipitation and delipidation, and with the Coomassie blue method without pretreatment. Similar results were obtained for rat bile.

Several conclusions regarding the utility of these three

assays for measuring total biliary proteins are justified from our data. First, use of either the Lowry or fluorescamine methods will provide accurate results only when bile is pretreated (e.g., by dialysis or TCA-precipitation and delipidation) to remove interfering substances. Under these circumstances, both assays will yield results similar to those obtained by amino acid analysis. Somewhat higher values are generally obtained by the Lowry method, perhaps because of residual interference by substances not totally removed or because of the presence in bile of specific types of proteins (e.g., those containing amino acids with phenol and hydroxyindole groups) which yield higher values by this method. Second, given the protein-to-protein variation which is apparent, particularly with the fluorescamine and Coomassie blue assays, a single standard should be consistently used. Considering our results and the fact that albumin is a major constituent of both rat and human bile, it would seem a suitable choice as standard for biliary protein assays. Third, the Coomassie blue method on native bile (i.e., without pretreatment) appears a reasonable choice for rat bile. However, despite the excellent correlation with results by amino acid analysis, in human bile the values are approximately two times higher than those obtained by amino acid analysis (Table 2, Fig. 3). Another disadvantage of the Coomassie blue method is that pretreatment such as TCA-precipitation or delipidation may actually interfere with the assay itself (Fig. 2). Thus, these maneuvers are not optimal pretreatments for the Coomassie blue assay. Regardless of the specific assay chosen, our results suggest that maneuvers to remove lipids, pigments, and/or free amino acids are important when measuring total biliary proteins in human gallbladder bile.

Total biliary proteins and gallstones

In Table 4, we have summarized published results for measurements of total protein concentrations on samples

TABLE 4. Values for total protein concentration in nonstone and stone bile

Author (year)	Ref. no.	Methods	Total Protein Concentration	
			Nonstone Bile	Stone Bile
			mg/ml	
Russell et al. (1964)	12	Immunodiffusion ^a	0.2 (n = 24)	0.2 (n = 14)
Arianoff and Heremans (1967)	13	Birthe-Tidström (bromophenol) ^b	2.6 (n = 10)	4.4 (n = 40) ^d
Holan et al. (1979)	9	Modified biuret ^a	4.6 (n = 7)	3.3 (n = 7) ^e
Sewell et al. (1983)	10	Lowry ^a	31.0 (n = 10)	17.5 (n = 44) ^f
Gallinger et al. (1987)	8	Fluorescamine ^b	1.5 (n = 34)	2.1 (n = 54) ^{e,g}
Yamazaki et al.	This study	Lowry ^b	5.6 (n = 10)	5.1 (n = 23) ^f
Yamazaki et al.	This study	Fluorescamine ^b	2.1	2.0
Yamazaki et al.	This study	Coomassie blue ^a	8.4	7.1
Yamazaki et al.	This study	Amino acid analysis ^b	3.6	3.1

^aNative bile.

^bPretreated bile.

^cCholesterol stone bile.

When comparing results for nonstone versus stone bile, ^d, $P < 0.01$; ^e, $P < 0.1$ (not significant); ^f, $P < 0.05$; ^g, $P < 0.0001$.

of gallbladder bile from subjects with and without gallstones, including also our own data. Several points are worth making. First, the number of studies in which total protein concentrations of human gallbladder bile have actually been measured is relatively small, reflecting, among other things, the fact that, until recently, samples of pure gallbladder bile could only be obtained at surgery. With the development of nonsurgical techniques for obtaining gallbladder bile via a transhepatic approach (41), the availability of such samples should increase. Including this report, however, four studies have been published just in the last 8 years, reflecting recent recognition of the probable importance of biliary proteins in the pathogenesis of cholesterol gallstones. Second, the reported concentrations of total protein in human gallbladder bile from subjects without gallstones have varied tremendously. However, when interfering substances are adequately removed, as was done in our study and in the report by Gallinger et al. (8), the concentration of total protein, regardless of the methods used for quantitation, falls within a fairly narrow range (i.e., from 1–8 mg/ml). Finally, published studies allow no confident conclusions regarding possible differences in total biliary protein concentration between subjects with and without gallstones; two studies showed no significant difference (9, 12), one reported higher concentrations in nonstone bile (10), and two reported higher concentrations in stone bile (8, 13). Our results using four different techniques suggest that patients with and without gallstones cannot be confidently separated on the basis of total biliary protein concentrations. We acknowledge, however, that we cannot exclude the possibility of a Type II error due to the relatively small number of nonstone and pigment bile samples. Interestingly, our results for total biliary protein in gallstone subjects using the fluorescamine assay were virtually identical to those reported by Gallinger et al. (8) who used the same assay after similar maneuvers to remove interfering substances (i.e., TCA-precipitation and delipidation). In contrast, our results in nongallstone subjects using this same technique are slightly higher than those reported by this group. Since similar criteria were used for subject selection, no explanation for this small difference is readily apparent.

While our results indicate no major differences in the concentrations of total biliary protein between subjects with and without gallstone, we do not interpret them as suggesting that biliary proteins are unimportant in the pathogenesis of cholesterol gallstones. Rather, they imply that efforts at defining the precise role of biliary proteins in cholesterol cholelithiasis would be better directed towards exploring quantitative and qualitative differences between nongallstone and gallstone subjects in individual biliary proteins (e.g., apolipoproteins, mucous glycoproteins), which are thought to be nucleation modifiers. In addition, attempts at developing in vitro functional assays

of cholesterol crystal nucleation and vesicle fusion would seem to be worthwhile. ■

We wish to thank Delia Pinon and Pamela Tietz for technical assistance, and Vel Woyczik for typing the manuscript. This work was supported by grants DK-24031 and DK-34988 from the National Institutes of Health, and by the Mayo Foundation. Some of this work has been previously published in abstract form (*Gastroenterology*. 1987. **92**: 1793; *Hepatology*. 1987. **7**: 1112).
Manuscript received 2 December 1987 and in revised form 26 February 1988.

REFERENCES

- Admirand, W. H., and D. M. Small. 1968. The physicochemical basis of cholesterol gallstone formation in man. *J. Clin. Invest.* **47**: 1043–1052.
- Holzbach, R. T., M. Marsh, M. Olszewski, and K. R. Holan. 1973. Cholesterol solubility in bile. Evidence that supersaturated bile is frequent in healthy man. *J. Clin. Invest.* **52**: 1467–1479.
- Carey, M. C., and D. M. Small. 1978. The physical chemistry of cholesterol solubility in bile. *J. Clin. Invest.* **61**: 998–1026.
- Holzbach, R. T. 1986. Recent progress in understanding cholesterol crystal nucleation as a precursor to human gallstone formation. *Hepatology*. **6**: 1403–1406.
- Lee, S. P., J. T. LaMont, and M. C. Carey. 1981. Role of gallbladder mucus hypersecretion in the evolution of cholesterol gallstones: studies in the prairie dog. *J. Clin. Invest.* **67**: 1712–1723.
- Holzbach, R. T., A. Kibe, E. Thiel, J. H. Howell, M. Marsh, and R. E. Hermann. 1984. Biliary proteins: unique inhibitors of cholesterol crystal nucleation in human gallbladder bile. *J. Clin. Invest.* **73**: 35–45.
- Kibe, A., R. T. Holzbach, N. F. LaRusso, and S. J. T. Mao. 1984. Inhibition of cholesterol crystal formation by apolipoproteins in supersaturated model bile. *Science*. **225**: 514–516.
- Gallinger, S., P. R. C. Harvey, C. N. Petrunka, R. G. Ison, and S. M. Strasberg. 1987. Biliary proteins and the nucleation defect in cholesterol cholelithiasis. *Gastroenterology*. **92**: 867–875.
- Holan, K. R., R. T. Holzbach, R. E. Hermann, A. M. Cooperman, and W. J. Claffey. 1979. Nucleation time: a key factor in the pathogenesis of cholesterol gallstone disease. *Gastroenterology*. **77**: 611–617.
- Sewell, R. B., S. J. T. Mao, T. Kawamoto, and N. F. LaRusso. 1983. Apolipoproteins of high, low, and very low density lipoproteins in human bile. *J. Lipid Res.* **24**: 391–401.
- Kawamoto, T., S. J. T. Mao, and N. F. LaRusso. 1987. Biliary excretion of apolipoprotein B by the isolated perfused rat liver. Relationship to receptor-mediated uptake of human low-density lipoprotein and biliary lipid secretion. *Gastroenterology*. **92**: 1236–1242.
- Russell, I. S., A. Fleck, and W. Burnett. 1964. The protein content of human bile. *Clin. Chim. Acta.* **10**: 210–213.
- Arianoff, A. A., and J. Heremans. 1967. Les protéines dans la bile humaine normale et pathologique. Etude quantitative. *Acta Gastro-Enterol. Belg.* **30**: 387–406.

14. Casu, A., D. Cottalasso, M. A. Pronzato, and A. M. Bassi. 1981. Study of proteins, phospholipids and cholesterol of rat bile during chronic drainage. *Ital. J. Biochem.* **30**: 388-402.
15. Kakis, G., and I. M. Yousef. 1978. Protein composition of rat bile. *Can. J. Biochem.* **56**: 287-290.
16. LaRusso, N. F., and S. Fowler. 1979. Coordinate secretion of acid hydrolases in rat bile: hepatocyte exocytosis of lysosomal protein? *J. Clin. Invest.* **64**: 948-954.
17. Billington, D., K. Rahman, T. W. Jones, R. Coleman, I. R. Sykes, and K. S. Aulak. 1986. Phospholipid degradation in, and protein content of, rat fistula bile. Contamination of bile with pancreatic juice. *J. Hepatol.* **3**: 233-240.
18. Soloway, R. D., B. W. Trotman, and J. D. Ostrow. 1977. Pigment gallstones. *Gastroenterology*. **72**: 167-182.
19. Tera, H. 1960. Stratification of human gallbladder bile in vivo. *Acta Chir. Scand. (Suppl.)* **256**: 4-85.
20. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**: 265-275.
21. Udenfriend, S., S. Stein, P. Böhlen, W. Dairman, W. Leimgruber, and M. Weigle. 1972. Fluorescamine: a reagent for assay of amino acids, peptides, proteins and primary amines in the picomole range. *Science*. **178**: 871-872.
22. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**: 248-254.
23. Russell, I. S., and W. Burnett. 1963. The proteins of human bile. *Gastroenterology*. **45**: 730-739.
24. Englert, E., Jr., E. E. Wales, and R. C. Straight. 1970. The proteins of human gallbladder bile with and without gallstones. *Clin. Chim. Acta.* **29**: 319-331.
25. Delacroix, D. L., H. J. F. Hodgson, A. McPherson, C. Dive, and J. P. Vaerman. 1982. Selective transport of polymeric immunoglobulin A in bile. Quantitative relationships of monomeric and polymeric immunoglobulin A, immunoglobulin M, and other proteins in serum, bile, and saliva. *J. Clin. Invest.* **70**: 230-241.
26. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*. **227**: 680-685.
27. Reeck, G. 1976. Amino acid compositions of selected proteins. In *CRC Handbook of Biochemistry and Molecular Biology, Section A: Proteins, Volume III*. G. D. Fasman, editor. CRC Press, Cleveland, OH. 504-519.
28. Hunter, W. M., and F. C. Greenwood. 1962. Preparation of iodine-131-labeled human growth hormone of high specific activity. *Nature*. **194**: 495-496.
29. Talalay, P. 1960. Enzymatic analysis of steroid hormones. *Biochem. Anal.* **8**: 119-143.
30. Roeschlau, P., P. Bernt, and W. Gruber. 1974. Enzymatic determination of total cholesterol in serum. *Z. Klin. Chem. Klin. Biochem.* **12**: 226-231.
31. Takayama, M., S. Itoh, T. Nagasaki, and I. Tanimizu. 1977. A new enzymatic method for determination of serum choline-containing phospholipids. *Clin. Chim. Acta.* **79**: 93-98.
32. Gurantz, D., M. F. Laker, and A. F. Hofmann. 1981. Enzymatic measurement of choline-containing phospholipids in bile. *J. Lipid Res.* **22**: 373-376.
33. Carey, M. C. 1978. Critical tables for calculating the cholesterol saturation of native bile. *J. Lipid Res.* **19**: 945-955.
34. McElderry, L. A., I. F. Tarbit, and A. J. Cassells-Smith. 1982. Six methods for urinary protein compared. *Clin. Chem.* **28**: 356-360.
35. Johnson, A., and J. A. Lott. 1978. Standardization of the Coomassie blue method for cerebrospinal fluid proteins. *Clin. Chem.* **24**: 1931-1933.
36. Jenzano, J. W., S. L. Hogan, C. M. Noyes, G. L. Featherstone, and R. L. Lundblad. 1986. Comparison of five techniques for the determination of protein content in mixed human saliva. *Anal. Biochem.* **159**: 370-376.
37. LaRusso, N. F. 1984. Proteins in bile: how they get there and what they do. *Am. J. Physiol.* **247**: G199-G205.
38. Clark, S. 1984. Determination of membrane protein concentration. In *Receptor Purification Procedures*. J. C. Venter and L. C. Harrison, editors. Alan R. Liss, Inc., New York. 149-161.
39. Tal, M., A. Silberstein, and E. Nusser. 1985. Why does Coomassie Brilliant Blue R interact differently with different proteins? *J. Biol. Chem.* **260**: 9976-9980.
40. Peters, W. H. M., A. M. M. Fleuren-Jakobs, K. M. P. Kamps, J. J. H. M. DePont, and S. L. Bonting. 1982. Lowry protein determination on membrane preparations: need for standardization by amino acid analysis. *Anal. Biochem.* **124**: 349-352.
41. Allen, M. J., T. J. Borody, T. F. Bugliosi, G. R. May, N. F. LaRusso, and J. L. Thistle. 1985. Rapid dissolution of gallstones by methyl tert-butyl ether. *N. Engl. J. Med.* **312**: 217-220.