UvA-DARE (Digital Academic Repository)

APF/CBP, the small, amphipathic, anionic protein(s) in bile and gallstones, consists of lipid-binding and calcium-binding forms

Lafont, H.; Domingo, N.; Groen, A.K.; Kaler, E.W.; Lee, S.P.; Koehler, R.; Ostrow, J.D.; Veis, A.

DOI

[10.1002/hep.510250502](https://doi.org/10.1002/hep.510250502)

Publication date

1997

Published in

Hepatology

[Link to publication](#)

Citation for published version (APA):

Lafont, H., Domingo, N., Groen, A. K., Kaler, E. W., Lee, S. P., Koehler, R., Ostrow, J. D., & Veis, A. (1997). APF/CBP, the small, amphipathic, anionic protein(s) in bile and gallstones, consists of lipid-binding and calcium-binding forms. *Hepatology*, 25, 1054-1063. <https://doi.org/10.1002/hep.510250502>

General rights

It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

Disclaimer/Complaints regulations

If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: <https://uba.uva.nl/en/contact>, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.

UvA-DARE is a service provided by the library of the University of Amsterdam (<https://dare.uva.nl>)

APF/CBP, the Small, Amphipathic, Anionic Protein(s) in Bile and Gallstones, Consists of Lipid-Binding and Calcium-Binding Forms

HUGUETTE LAFONT,¹ NICOLE DOMINGO,¹ ALBERT GROEN,² ERIC W. KALER,³ SUM P. LEE,³ RONALD KOEHLER,⁴ J. DONALD OSTROW,^{2,4} AND ARTHUR VEIS⁵

Two very similar small anionic, amphipathic proteins, a phospholipid-binding apoprotein (anionic polypeptide fraction [APF]) and a calcium-binding polypeptide (CBP), are found abundantly in bile and all types of gallstones. The often disparate properties among various preparations of APF/CBP could reflect different sources and separation procedures, leading to partly degraded and/or denatured protein and varied association of bile salts, lipids, bile pigments, and detergents. The present study presents new methods for isolation and purification of APF/CBP, and characterizes the preparations thus obtained. It was found that isolation by selective precipitation of proteins from fresh T-tube bile by added calcium chloride, followed by demineralization with ethylenediaminetetraacetic acid (EDTA), removal of salts, lipids, and some pigment by Sephadex LH-20, and serial ultrafiltration yields the purest preparations. Though free of lipids, bile salts, detergents, and most pigments, these new preparations all show the same 7-kd and 12-kd bands on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), the same major peaks on hydrophobic high-performance liquid chromatography (HPLC), and retain the self-associative, lipid- and calcium-binding functions, typical of older preparations obtained by potentially denaturative procedures. The varied properties among APF/CBP preparations are thus apparently related mainly to their content of different proportions of two major components, lipid-binding APF and calcium-binding CBP. Immunologic cross-reactions indicate common epitopes, and amino acid analyses are also similar, suggesting that APF and CBP may have the same polypeptide backbone, but differ because of posttranslational modification(s). Sufficiently pure APF and CBP have now been obtained to permit possible

structural identification by sequencing and molecular biological techniques, though such attempts have thus far been unsuccessful. (HEPATOLOGY 1997;25:1054-1063.)

Anionic polypeptide fraction (APF) and calcium-binding protein (CBP) are two very similar, small, amphipathic, highly anionic proteins that are present in high concentrations in both normal and pathological bile and all types of gallstones.¹⁻³ APF, the phospholipid-binding apoprotein of the bile pigment–lipoprotein complex of bile,^{4,5} is involved in the secretion of cholesterol and phospholipids into bile.⁶ CBP binds calcium tightly and promotes the precipitation of calcium salts when bound to mucin,⁷ but inhibits calcium salt precipitation when not bound to mucin.⁷⁻⁹ APF, however, is also found in gallstones,^{10,11} where it is uniquely localized to the pigmented zones, interposed between the mucin matrix and the calcium-pigment deposits.¹¹ These properties suggest that APF and/or CBP may play key roles in the regulation of the precipitation of calcium salts from supersaturated bile, and the deposition of these salts on the structural mucin matrix of the gallstones.^{1,2,11}

Neither APF nor CBP has been fully purified or sequenced, and the various preparations from different laboratories,^{4,5,7-13} isolated from different sources using different procedures, often exhibit disparate properties, even though all display 12-kd as well as 7-kd bands on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), and all exhibit immunologic cross-reactivities and very similar amino acid compositions.¹³ The present collaborative communication from these laboratories presents new isolation methods and compares the properties of these preparations with those of older preparations, especially their interactions with bile pigments and calcium. A final hydrophobic high-performance liquid chromatography (HPLC) step separates even these more highly purified APF/CBP preparations into several apparently pure protein peaks, only some of which avidly bind calcium. The results suggest that differences among the various APF/CBP preparations result from: 1) varied contents of lipids, bile salts, pigments, and detergents, and 2) varied proportions of lipid-binding (APF) and calcium-binding (CBP) proteins. The nature of the associated pigments, the relationships of the 7-kd and 12-kd bands on SDS-PAGE, and future approaches to purification, are also discussed.

MATERIALS AND METHODS

Preparations of APF/CBP Previously Published. APF-but was isolated from human bile obtained by intraoperative gallbladder puncture during cholecystectomy for cholesterol gallstones^{2,4,5,13} by ultracentrifugation in a sucrose density gradient, followed by dialysis, lipid extraction with *n*-butanol, and ultrafiltration through a Centricon-10 device (Amicon, Danvers, MA).

CBP-Chi was prepared from the pigmented shells of human cholesterol gallstones, as described previously,^{8,9,13} by sequential extrac-

Abbreviations: APF, anionic polypeptide fraction; CBP, calcium-binding protein; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; HPLC, high-performance liquid chromatography; EDTA, ethylenediaminetetraacetic acid.

From the ¹Lipid Research Laboratory, INSERM U-130, Marseille, France; ²Liver/Gastrointestinal Department, Academic Medical Center, Amsterdam, the Netherlands; ³Gastroenterology Service, DVA Medical Center and Department of Medicine, University of Washington, Seattle, WA; ⁴Research Service, DVA Lakeside Medical Center and Gastroenterology/Hepatology Division, Department of Medicine, and ⁵Department of Oral Biology, Northwestern University Medical School, Chicago, IL.

Received January 15, 1996; accepted January 31, 1997.

Supported by INSERM, France (Dr. Lafont); the Netherlands Liver and Digestive Disease Foundation (NLDS) (Drs. Groen and Ostrow); Merit Review Research Awards (Drs. Lee and Ostrow) and a Medical Investigator Award (Dr. Ostrow) from the U.S. Department of Veterans Affairs; and Research Grant 2-RO1-DK-32130 from the NIDDK, National Institutes of Health, Bethesda, MD (Drs. Ostrow and Veis).

Address inquiries to: Prof. Dr. J. Donald Ostrow, Room C2-111, M.D.L., Academic Medical Center, Meibergdreef 9, 1105 AZ Amsterdam Z.O., The Netherlands. Fax: 31-(20)-691-7033.

Copyright © 1997 by the American Association for the Study of Liver Diseases.
0270-9139/97/2505-0003\$3.00/0

tion with methyl-*t*-butyl ether, methanol, and disodium ethylenediaminetetraacetic acid (EDTA), followed by column chromatography on Sepadex G-25 and two-step preparative SDS-PAGE in a Schagger-von Jagow gel.¹⁴ A revision of this method⁷ yielded *CBP-xol* from the body of "pure" human cholesterol gallstones, *CBP-mix* from mixed cholesterol gallstones, *CBP-pig* from the pigmented shells of cholesterol stones, *CBP-brn* from brown pigment stones, and *CBP-S&P* from salt-and-pepper stones. The procedure was the same as for *CBP-Chi*, with three key modifications⁷: preparative SDS-PAGE on 15% Laemmli gel,¹⁵ electroelution without SDS, and removal of most of the SDS by precipitation with 50 mmol/L KCl.¹⁶

CBP-new was prepared from bile by precipitation with calcium,¹³ followed by treatment of the precipitate in the same manner as gallstone powder, as described for *CBP-xol*.⁷

Newest Procedures to Minimize Protein Denaturation During Isolation. *APF-Mar* was prepared from centrifuged (15,000g for 30 minutes) gallbladder bile from a patient with cholesterol gallstones by serial ultrafiltration through Minultrasette devices (Filtron, Coignières, France), avoiding denaturation by organic solvents and SDS. The bile supernatant was processed successively through 100-kD, 30-kD, and 10-kD filters, saving each ultrafiltrate for the next step. The 10-kD filtrate was then concentrated by ultrafiltration through a hydrophobic Omega membrane (3.5-kD) (Filtron) that had been presoaked overnight in glycerol, and the concentrated retentate was stored at -80°C; this final step removed remaining salts, bile salts, and lipids.

CBP-Sea was prepared from intraoperative gallbladder bile, or from T-tube bile obtained postcholecystectomy, from patients with cholesterol gallstones, by a modification that omitted the solvent extraction steps from a previously described procedure.¹² After precipitation of proteins from centrifuged bile with calcium carbonate, the dried precipitate was demineralized with disodium EDTA. The EDTA extract was dialyzed in a Spectra-Por 3.5-kD membrane (Spectrum, Houston, TX) at 4°C against distilled water for 24 hours. The protein was recovered by lyophilization without prior Sephadex treatment (crude *CBP-Sea*), and finally purified by hydrophobic HPLC (see below).

Analytical SDS-PAGE. Analytical SDS-PAGE was performed in a 16.5-T, 3C Schagger-von Jagow gel,¹⁴ and also in a 15% Laemmli gel,¹⁵ each 0.75 mm thick. The full gels were stained with silver¹⁷ using the Bio-Rad (Richmond, CA) kit, and counterstained with Coomassie blue. Gels were dried and scanned with a GS-670 densitometer (Bio-Rad) at 400 to 750 nm, and the data were converted to a graphic representation using the Molecular Analyst 1.1 program (Bio-Rad).

Hydrophobic HPLC. Hydrophobic HPLC¹⁸ was performed with two systems; for both, detection was at 280 nm, and the column was used without a prefilter and was washed once daily with isopropanol/ Na_2HPO_4 (0.02 mol/L, pH 7.4) (4:1 vol/vol), and then pre-equilibrated with the relevant $(\text{NH}_4)_2\text{SO}_4$ buffer.

In Seattle, a 7.5 × 75-mm HIC-5PW column (Biorad, Hercules, CA) was developed at a flow rate of 3.5 mL/min with a multistep gradient: 0 to 15 minutes, isocratic $(\text{NH}_4)_2\text{SO}_4$, 0.75 mol/L (buffer B), adjusted to pH 7.0; 15 to 30 minutes, a linear gradient from 100% buffer B to 100% Na_2HPO_4 (0.02 mol/L, pH 7.4) (buffer A); 30 to 40 minutes, 100% buffer A; 40 to 50 minutes, a linear gradient from 100% buffer A to 100% buffer B; 50 to 75 minutes, 100% buffer B. Protein, approximately 1.0 mg dry weight dissolved in buffer B, was applied to the column, and 3.5-mL fractions were collected each minute for 75 minutes.

In Marseille, a 7.5 × 75-mm Spherogel TSQ Phenyl 5 PW Cellufine column (Beckman, Paris, France) was used. It was developed with a linear gradient from 100% $(\text{NH}_4)_2\text{SO}_4$ (1.7 mol/L, adjusted to pH 7.4), to 100% Na_2HPO_4 buffer (20 mmol/L, pH 7.4) over 30 minutes, followed by 5 minutes at 100% Na_2HPO_4 buffer. Between runs, the column was washed with a gradient of 100% methanol/water (1:1 vol/vol) to 100% $(\text{NH}_4)_2\text{SO}_4$ (1.7 mol/L, pH 7.4) over 30 minutes, followed by 5 minutes of the $(\text{NH}_4)_2\text{SO}_4$ buffer. Protein, 200 to 500 mg dissolved in the $(\text{NH}_4)_2\text{SO}_4$ buffer, was applied and 1.0-mL fractions were collected for 45 minutes at a flow rate of 1.0 mL/min.

Calcium Binding. Calcium binding was modified from Kestell et al.¹² Distilled, deionized water was used for all procedures. Protein (30–50 µg in 5.0 µL water) was adsorbed to a 13-mm-diameter PVDF membrane (Immobilon-P, Millipore, Bedford, MA) that had been successively prewashed for 10 minutes each with methanol, water (5×), 2N HCl, and water (5×). Binding of $^{45}\text{Ca}^{++}$ was assessed by incubat-

ing the membrane-bound protein for 20 minutes at $21 \pm 1.5^\circ\text{C}$ in $^{45}\text{CaCl}_2$ (19.8 mCi/mg) (New England Nuclear, Billerica, MA) dissolved in water (2 µCi/mL). After washing twice with water, bound $^{45}\text{Ca}^{++}$ was assayed by liquid-scintillation spectrometry. Each coded protein sample was run in triplicate, with calmodulin (5 mg) as a strongly binding control and bovine serum albumin (25 mg) as a weakly binding control (both from Sigma, St. Louis, MO). All counts were corrected by subtracting the background of 7,657 dpm of $^{45}\text{Ca}^{++}$ bound by the membrane alone.

Effects of APF/CBP Preparations on Precipitation of Calcium Phosphate. The effects of APF/CBP preparations were studied according to Afidhal et al.,⁷ using the seeded system.

Azopigments. Azopigments of APF/CBP preps were prepared by a modification of the method of Trotman et al.¹⁹ The Sephadex G-25 fraction from *CBP-pig* or *CBP-xol* (400 mg), or 100 mg of the 7-kD or 12-kD bands of *CBP-pig* eluted from a Laemmli preparative gel, was dissolved in 0.1 mL 0.15 N NaCl plus 1.0 mL dimethyl sulfoxide. After reaction with 0.4 mL diazotized ethyl anthranilate for 30 minutes at 4°C, fresh diazo-reagent was added and the reaction continued for another 30 minutes. Ascorbic acid and 1.0 mL glycine-HCl buffer, pH 2.7, were added, and azopigments were extracted twice into 2-pentanone:butyl acetate (17:3 vol/vol). The pooled organic phase was washed five times with glycine-HCl buffer to remove dimethyl sulfoxide and residual diazo-reagent, and residual glycine and salts were removed from the aqueous phase by 10× ultrafiltration through a 3-kD Centricon device, rediluting the retentate with 9 vol water each time. Washed organic and aqueous phases were examined spectroscopically (360–700 nm), and then each was lyophilized in two aliquots. One aliquot was dissolved in methanol-water (1:1 vol/vol), and azopigments were separated by thin-layer chromatography on silica gel,²⁰ developed with chloroform:methanol:water (60:20:10 by vol), using azopigments prepared from rat bile as standards. The other aliquot was dissolved in sample buffer and subjected to SDS-PAGE on both Laemmli and Schagger-von Jagow gels.

Amino Acid Analyses. Amino acid analyses were performed as described,^{8,13} using an automated amino acid analyzer with ninhydrin detection (Beckman 6300, Paris, France). Hydrolysis was performed for 22 hours under a vacuum at 118°C with 5.6 N HCl, as well as with mercaptoethane sulfonic acid (Pierce, Paris, France) to preserve tryptophan.²¹ After evaporation of HCl or adjustment to pH 2.0 with NaOH, dried residues were dissolved in sodium citrate buffer (2% wt/vol, pH 2.0) for injection into the analyzer.

Analyses of Lipids and Bile Salts. Analyses of lipids and bile salts were performed using up to 1.0 mg of APF/CBP preparations. Phosphatidylcholine was estimated with an enzymatic kit (Boehringer, Mannheim, Germany).²² Total bile acids were estimated by a fluorimetric modification of the 3α-hydroxysteroid dehydrogenase assay,²³ with reagents from Boehringer-Mannheim. Lipid and bile salt compositions were analyzed by thin-layer chromatography of the proteins on silica gel (Alufolien Kieselgel 60F-254, Merck). Bile salts and phospholipids were developed with chloroform:methanol:water (65:25:4 by vol),²⁴ and neutral lipids were developed with heptane:diethyl ether:acetic acid (90:30:1 by vol).²⁵ Appropriate standards (A grade; Sigma) were run in parallel lanes, and bands were identified by spraying with phosphomolybdate reagent.

Other Chemical Analyses. Protein concentrations were measured with the Pierce bicinchonic acid method,²⁶ adjusted for a standard curve prepared with bovine serum albumin (Fraction V; Sigma). SDS concentrations were measured by a modification of the method of Sharma et al.,²⁷ in which extraction was performed with pH 5.0 citrate buffer and with only 0.002 NH_2SO_4 in the methylene blue solution to avoid precipitation of APF/CBP that occurred at lower pH values.

APF/CBP Antibodies. Peak 4 from the HPLC of *APF-Mar* was used as antigen to prepare a specific polyclonal antiserum in rabbits (PAb-APF-94), by methods described previously. Previously reported monoclonal antibodies (MAB-APF-32) from BALB/c mice, were also used.¹³

Antibody Titers. Antibody titers were assessed by direct enzyme-linked immunosorbent assay.¹³ After coating wells with 0.25 to 3.0 mg APF or CBP, nonspecific reactions were blocked by coating the plates with gelatin digest (Boehringer-Mannheim) before incubation with antibodies. The immune complexes were identified with anti-rabbit immunoglobulin G coupled to horseradish peroxidase, using TMB- H_2O_2 as substrate, or to alkaline phosphatase, using *p*-nitrophenyl phosphate as substrate, as directed by the manufacturer (Sigma, Paris, France).

RESULTS

Color (Pigment). Final, dried CBP preparations from gallstones all were brown in color, in rough proportion to the amount of pigment in the source gallstones. The intensity ranged from light brown for *CBP-xol* to very dark brown for *CBP-Chi*. Among preparations derived from bile, in contrast, bile pigment-lipoprotein complex and crude *CBP-Sea* were green, whereas *APF-but*, *APF-Mar*, *CBP-new*, and purified *CBP-Sea* were only faintly yellow-green.

SDS, Lipids, and Bile Salts. *CBP-xol* (and related CBP preparations isolated from gallstones by the same method) contained less than 0.4 mol SDS per mol protein, but, like *APF-but*, were free of lipids and bile salts. Crude *APF-Mar* and crude *CBP-Sea*, contained some bile salts and phospholipids. All hydrophobic HPLC peaks from all preparations were free of detectable bile salts and lipids.

SDS-PAGE. In the Schagger-von Jagow, tricine-buffered gel system, all APF and CBP preparations yielded mainly green to brown bands, of average apparent molecular weight ranging from 10 to 14 kd and 6 to 8 kd (hereafter called, respectively, the 12-kd and 7-kd bands). The more highly pigmented preparations usually exhibited more intense silver staining, as well as a brown to green "wash" from about 16 to 5 kd, which also stained with silver, partly obscuring the principal bands. In the Laemmli gels, by contrast, most of the pigment separated from the protein bands and ran ahead of the bromophenol blue front, and the two major protein bands ran at 7.5 to 6.0 kd and 4.5 to 5.0 kd.

For each CBP preparation, the two major bands, eluted from a preparative Schagger-von Jagow or Laemmli gel and rerun on a second-stage Schagger-von Jagow analytical gel, yielded mainly the same band in the same range of apparent molecular weight (Fig. 1A and B; lanes 1 and 2 of Fig. 2A and B); small proportions of the lower band were sometimes found in the rerun of the upper band because of overlap in the first-stage gel. In both systems, the less-pigmented preparations (e.g., *CBP-mix*, *CBP-xol*, *CBP-Sea*, and *APF-but*) generally yielded mainly the 12-kd band, whereas the more heavily pigmented preparations (*CBP-pig* and *CBP-brn*) yielded mainly the 7-kd band. The newer *APF-Mar* and *CBP-new* preparations yielded only the 7-kd band (not shown). Fainter silver-stained bands of molecular weight 67, 18, and below 3 kd, often seen in the first-stage gels, were absent in all second-stage gels. The same SDS-PAGE patterns were seen with or without mercaptoethanol in the buffers, but native PAGE (no SDS) in either type of gel yielded a long pigment smear throughout each lane, which revealed no banding on silver stain (not shown).

Diazotization. The lightly pigmented Laemmli gel bands of *CBP-xol* and *APF-Mar* yielded only traces of azopigment in the organic extract. In contrast, *CBP-pig* yielded azopigments in the organic phase equivalent to approximately 1.2 mol bilirubin per mol protein. This ran as the unconjugated α -azopigment on thin-layer chromatography (not shown), at <2.0 kd on a Schagger-von Jagow SDS-PAGE gel (Fig. 2A, lane 4) and ahead of the front on a Laemmli gel (not shown). With all preparations, only traces of azopigment remained in the aqueous phase; these did not migrate from the origin on thin-layer chromatography, but yielded a protein band at 6.0 to 8.5 kd (Fig. 2A, lane 5). The more heavily pigmented stone and gallbladder bile preparations also yielded a precipitate at the interface, which was yellow-brown without any visible purple color typical of azopigments. On SDS-PAGE, this redissolved precipitate yielded a broad, yellow-brown smear that stained with silver (Fig. 2A and B, lane 3).

Hydrophobic HPLC. Using the Seattle system, *APF-but* yielded a minor peak at 13 minutes with a long tail, and a major, broad, composite peak at 42 minutes with shoulders

at 49 and 57 minutes (Fig. 3A). By contrast, crude *CBP-Sea* yielded three tall, sharp, but overlapping, peaks, 1a-c, at 5.2, 6.5, and 7.5 minutes, as well as a symmetrical, shallower, but broad, peak 2 at about 40 minutes (Fig. 3B). The eluted peak 1b at 6.5 minutes (purified *CBP-Sea*) was dialyzed, lyophilized, and subsequently rechromatographed in the Marseille system.

Using the simpler Marseille gradient, each preparation except purified *CBP-Sea* yielded four major peaks (Fig. 4A-E; Table 1). The slowest and most heavily pigmented peak ran at 30 to 33 minutes with *APF-Mar* (Fig. 4A, peak 4) and at 33 to 40 minutes with *CBP-xol* 7-kd and 12-kd and crude *CBP-Sea* (Fig. 4B-D, peak 5). In each case, the two sharp, closely spaced peaks 2 and 3 were least abundant (by optical density at 280 nm), but varied in their amounts relative to each other. The 7-kd and 12-kd bands of *CBP-xol* yielded very similar patterns, with a relative paucity of peak 1 (Fig. 4B and C). Purified *CBP-Sea* uniquely yielded only a broad, lightly pigmented peak 1 and a sharp, colorless peak 3 (Fig. 4E).

Calcium Binding. Corrected dpm of $^{45}\text{Ca}^{++}$ bound per milligram of protein were: calmodulin, 31,702; purified *CBP-Sea*, 17,293; *CBP-new*, 7,442; *APF-but*, 893; hydrophobic HPLC peak 4 from *APF-Mar*, 70; and bovine serum albumin, <100.

Effects on Calcium Phosphate Precipitation. Like *CBP-pig*, *CBP-brn*, *CBP-S&P*, and *CBP-mix* reported previously,⁷ *CBP-xol*, *CBP-new*, and *CBP-Sea* also inhibited the precipitation of calcium phosphate, and this inhibition was either eliminated or converted to promotion of precipitation when the CBP was prebound to bovine gallbladder mucin. As expected, *APF-but* and *APF-Mar* did not affect calcium phosphate precipitation, either with or without mucin present.

Enzyme-Linked Immunosorbent Assays of Hydrophobic HPLC Peaks, Using Pab-APF-94. Among peaks eluted from the Marseille system (Table 1), peaks 2 and 3 showed the greatest reactivity per milligram of protein, especially peak 2. Peaks 1 and 4 showed weaker reactivity (less than 25% of that of peaks 2 and 3). Peak 5 was minimally reactive, possibly because the large amount of pigment in this peak led to overestimation of the amount of protein based on an optical density of 280 nm. Despite their lower immunoreactivity, the much more abundant peaks 1 and/or 4 or 5 accounted for more than half of the reactivity of *APF-Mar* and both crude and purified *CBP-Sea*. Reactivity of individual peaks agreed well among the different APF/CBP preparations.

Amino Acid Analyses. The 7- and 12-kd bands of *CBP-new* from bile showed closely similar patterns (Table 2). Dicarboxylic amino acids constituted 22% to 23% of residues with more glutamate than aspartate. Dibasic amino acids constituted about 9% of residues, with Lys > Arg > His. Glycine constituted only 11% of residues, and methionine constituted 2%, but cysteine was not detected. *APF-Mar* showed a similar pattern, except for a higher content of Gly, a lower content of Tyr, the presence of some Cys, and the absence of Met.

The 12-kd band of *CBP-xol* and the 12-kd and 7-kd bands of *CBP-mix* were likewise similar to each other, but differed modestly from the patterns in *CBP-new*, showing no Met, less Tyr, and more Asp, Gly, and possibly Arg. These three stone proteins also showed a higher proportion of total diacidic amino acids, and a greater excess of diacid over dibasic amino acids, than the bile proteins. The absence of Trp in all preparations was confirmed separately after nondestructive hydrolysis.²⁸

DISCUSSION

Isolation and purification of the phospholipid and calcium-binding proteins from gallstones and bile are complicated by the strong interactions between these proteins and the bile salts, lipids, and pigments also present.¹ The concentrations of these components vary from one bile or stone sample to

FIG. 1. Second-stage SDS-PAGE, in Schagger-von Jagow 16.5T, 3C system, of bands electroeluted from preparative 15% Laemmli gels. Each CBP preparation was isolated from gallstones by the revised method of Afdhal et al.,⁷ and 20 μ g of protein was applied from each electroeluted Laemmli gel band. Bio-Rad silver stain with Coomassie blue counterstain. Very-low molecular weight standards are in the extreme left lane, and the medium-molecular-weight standards are in the extreme right lane (molecular weights are in kd). (A) *CBP-pig*. Lanes: 1, 12 kd; 2, 7 kd; 3, 3.5 kd; 4, 2.5 kd; and 5, < 2 kd. (B) Lanes: 1 and 2, *CBP-mix*; 3 and 4, *CBP-S&P*; and 5 and 6, *CBP-brn*. The 12-kd bands are in the odd-numbered lanes and the 7-kd bands are in the even-numbered lanes.

another; thus, in a sense, the protocols for preparation of the proteins must be adjusted almost on an individual basis. Further complicating the isolation of the two protein components are their apparent similarity in molecular size and composition, and their strong hydrophobic interaction to form higher mass complexes^{1,12}; thus, APF and CBP tend to collect in the same fractions throughout most stages of the isolation procedures.

The present study was performed primarily to understand the differences among various APF/CBP preparations and to use this information to develop better isolation and purification procedures for each protein component. A second aim was to better characterize the differences and relationships

between APF and CBP, as well as the 7-kd and 12-kd bands seen on SDS-PAGE of these proteins. In view of the often conflicting literature, we organized a collaborative effort among five laboratories to compare the preparations obtained following their different protocols.

Characteristics of the Different APF/CBP Preparations

Preparations Obtained by Older Published Methods (APF-but and CBP-Chi). APF preparations, isolated from bile by sucrose density gradient centrifugation,^{4,13} contained no SDS, but were significantly associated with amphipathic components (phospholipids, pigment, and bile salts) of the bile-lipoprotein complex. These amphipaths were essentially com-

FIG. 2. Second-stage SDS-PAGE, in Schagger-von Jagow 16.5T, 3C system, of bands electroeluted from preparative 15% Laemmli gels, and of azopigments prepared from the 12-kd bands. (A) *CBP-pig* and (B) *CBP-xol*, each isolated from gallstones by the revised method of Afdhal et al.⁷ Bio-Rad silver stain with Coomassie blue counterstain. Very-low molecular weight standards (L) are in the extreme left lane (molecular weights are in kd). Protein (20 μ g) was applied from the electroeluted 12-kd Laemmli gel band (lane 1) and the 7-kd band (lane 2). The redissolved protein from the diazo-reaction precipitate is in lane 3. In (A), lane 4 is the organic phase extract, and lane 5 is the aqueous phase from the diazo-reaction; lane 6 is empty, and medium-molecular-weight standards are in the extreme right lane. This gel was divided between lanes 3 and 4, allowing parallel, more prolonged silver-staining of lanes 4 and 5 to visualize the bands, which were barely stained, and, therefore, not shown in (B).

pletely removed by extraction with butanol or butanol-ether (*APF-but*). CBP preparations, isolated from gallstones by solvent extraction and demineralization, followed by preparative SDS-PAGE in the Schagger-von Jagow system (*CBP-Chi*), contained considerable pigment and SDS, and some bile salts, but no phospholipids.

Preparations Obtained by the Revised Methods (*CBP-xol*, *CBP-mix*, *CBP-brn*, *CBP-S&P*, *CBP-pig*, and *CBP-new*). In contrast with the Schagger-von Jagow gel, the Laemmli gel system releases of most of the pigment from CBP. This may

be due to the lower pH of the Laemmli gels, resulting in less ionization of the bilirubin and, consequently, less affinity for the protein, as occurs with albumin.²⁹ The Schagger-von Jagow gel, however, gives better analytical definition of very-low-molecular-weight polypeptides,¹⁴ such as *APF/CBP*. Both electroelution without SDS and KCl precipitation of most residual SDS minimized, but did not eliminate, this detergent from the final preparations. Complete avoidance of SDS, by use of native PAGE for protein and pigment separation, is precluded by the marked self-aggregation of CBP.¹²

FIG. 3. Hydrophobic HPLC patterns in the Seattle system 1, with detection at 280 nm. (A) *APF-but*, showing mainly a multicomponent hydrophobic peak. (B) Crude *CBP-Sea*, showing sharp, partially overlapping peaks 1a, b, and c, at 4 to 10 minutes, and a broad, more hydrophobic peak 2, centered at 40 minutes. The tallest, middle peak 1b of *CBP-Sea* was processed further in the Marseille HPLC system (see Fig. 4E).

Preparations Obtained by the Newest Methods, Without Use of Lipid Solvents or SDS. Because the APF/CBP complex is the major protein precipitated from bile by the addition of CaCl_2 and relatively little cholesterol co-precipitates, this approach provided a simple, quick, and quite selective purification step, free of the prolonged, harsh solvent extractions required to remove cholesterol from the gallstone powders. Lipids and bile salts remained, however, if the Sephadex gel filtration was omitted, as with crude *CBP-Sea*.¹² Serial ultrafiltration of bile (*APF-Mar*) only partially removed lipids, and, because of self-aggregation, over 80% of the APF/CBP was lost in the discarded 10-kD retentate, as revealed by subsequent SDS-PAGE (data not shown). Almost all the remaining lipids, but only part of the pigment, were removed from *APF-Mar* by a final ultrafiltration through a 3.5-kD Omega membrane.

Bile Pigments. The noncovalently bound pigment, which was largely removed by the Laemmli SDS-PAGE, was diazo-reactive and formed mostly unconjugated azopigments. Thus, even though bilirubin conjugates are the dominant pigments in bile,³⁰ APF/CBP, like albumin,²⁹ preferentially bound unconjugated bilirubin. The residual pigment, which was apparently covalently bound, was present in rough proportion to the degree of pigmentation of the source gallstones, and was least evident in APF or CBP isolated from fresh hepatic bile. The prolonged sojourn of a gallstone or bile in the gallbladder would permit more extensive trans-esterification of bilirubin glucuronides to lysine- NH_2 groups on the proteins,^{31,32} as well as degradation of the proteins. Therefore, uninfected T-tube

bile, freshly collected in the dark into an iced receptacle containing protease inhibitors, should be the preferred source for isolation of APF/CBP.

The residual pigment reacted minimally with the diazo-reagent, possibly because it was a pigment polymer,³³ biliverdin, or porphyrin, which lack the saturated central methylene bridge required for diazo-reactivity.³⁴ Alternatively, this reactive group might have been sterically inaccessible within the three-dimensional structure of the protein. The increased electrophoretic mobility of the 12-kD band after diazotization (Fig. 2A, lanes 1 and 5) may be caused by extraction of associated hydrophobic substances by the solvents for the diazo-reaction.

Amino Acid Compositions. Except for *CBP-Sea*,¹² which contained almost twice as much Pro and Leu and almost 15% dibasic amino acids (with Arg = His), the amino acid compositions of the newer preparations generally resembled the published analyses of APF/CBP prepared by older methods.¹³ Polypeptide fragments in the large HPLC peak 1 of crude *CBP-Sea* (Fig. 4D) might account for its discrepant pattern, which exhibited a much smaller excess (7%) of diacidic as compared with dibasic amino acids. All the other preparations showed a 14% to 17% excess of diacidic as compared with dibasic amino acids, accounting for the low pI .¹³

The presence of almost 2 residue% Met in *CBP-new* obtained from fresh bile by calcium precipitation suggests that the absence of Met in CBPs prepared from gallstones or gallbladder bile resulted from degradation during their prolonged residence in the gallbladder. The remaining 50% of amino acid residues are primarily neutral and hydrophobic, and account for the strongly amphipathic character of APF and CBP and their marked tendency to aggregate and to bind other amphipaths, such as bile salts, phospholipids, and pigments.^{1,2} The hydroxyl groups in the abundant Thr + Ser residues in all APF/CBP preparations may cooperate with the $-\text{COO}^-$ sidechains in the binding of Ca^{++} ions.³⁵

Immunoreactions

Though pigment may function as an antigenic haptene in bilirubin-protein conjugates,³⁶ this is probably not the case with APF/CBP. Thus, the lightly pigmented peaks 2 and 3 in *CBP-xol* 12-kD and pure *CBP-Sea* were strongly immunoreactive, whereas the highly pigmented peak 5 in *CBP-xol* and crude *CBP-Sea* showed little immunoreactivity (Table 1).

The calcium-binding HPLC peaks 2 and 3 had great immunoreactivity against PAb-APF-94, despite the fact that this antibody was prepared against HPLC peak 4 from *APF-Mar*. Because MAb-32 also reacted with both components of APF/CBP (data not shown), the two proteins very likely share common epitopes.

Features of Newer Purification Methods, and Nature of HPLC Peaks

CBP-xol, *CBP-new*, and *APF-Mar* contained less of the non-protein components, and the latter two, prepared from fresh bile, are probably less degraded than previously reported APF/CBP preparations. None of the newer APF/CBP preparations were pure, however, because hydrophobic HPLC revealed that each contained two major components and several minor components (Fig. 4). Fortunately, hydrophobic HPLC both fractionated the proteins and separated any remaining lipids and most of the pigment. The residual pigment appeared mainly in HPLC peaks 4 or 5, and probably contributed to the long retention times of these more hydrophobic fractions. Peak 5 showed negligible immunoreactivity and contained little protein; most of its optical density at 280 nm was, therefore, probably related to pigment. Purified *CBP-Sea* contained little pigment and no detectable peak 5.

FIG. 4. Hydrophobic HPLC patterns in the Marseille system with detection at 280 nm. (A) *APF-Mar*; (B) *CBP-xol* 7-kd band; (C) *CBP-xol* 12-kd band; (D) crude *CBP-Sea*; and (E) purified *CBP-Sea* (calcium-binding peak 1b from Fig. 3B).

Preparative SDS-PAGE virtually eliminated HPLC peak 1 from *CBP-xol* (Fig. 4B and C), but it was prominent in the other preparations, whether isolated by calcium precipitation (*CBP-Sea*) or serial ultrafiltration (*APF-Mar*). The immuno-

reactivity of this compound peak suggests that it consisted of polypeptide fragments and/or aggregates of *APF/CBP*, some of which contained reactive epitopes. Peak 1 was not removed by the simple method of serial ultrafiltration (*APF-*

TABLE 1. Characteristics of Hydrophobic HPLC Peaks From APF/CBP Preparations

Preparation: μg of Protein Recovered/Applied	Peak No. RT, min.	1 3-6	2 12-13	3 14-15	4 30-33	5 33-40
APF-Mar 400/500	protein* pigment† ELISA‡	233, broad ++ 1.0	19, sharp + not tested	24, sharp + 5.8	500, sharp ++ 1.6	not seen
CBP-xol, 7kd 231/400	protein* pigment† ELISA‡	20, broad + 1.4	36, sharp ++ 7.9	19, sharp ++ 5.4	not seen	298, broad +++ not tested
CBP-xol, 12 kd 199/400	protein* pigment† ELISA†	19, broad + not tested	14, sharp + 7.5	15, sharp + 6.6	not seen	216, broad ++ 0.1
CBP-Sea, Crude 307/400	protein* pigment† ELISA‡	50, broad + not tested	15, sharp + 7.0	20, sharp + 4.7	not seen	908, broad ++++ 0.1
CBP-Sea, Purified 77/200	protein* pigment† ELISA‡	160, broad ++ 1.2	not seen	58, sharp none 3.0	not seen	not seen

NOTE. Using Marseille system for hydrophobic HPLC, developed with a single linear gradient. See text and Fig. 4 for details.

* Protein: computer-derived area under the curve of peak at 280 nm (AUC_{280 nm}); shape of peak.

† Pigment: assessed from visual yellow color of eluted fraction.

‡ ELISA: reactivity to polyclonal antibody against APF-Mar peak 4 (PAb-APF-94) by enzyme-linked immunosorbant assay (O.D._{ELISA}/AUC_{280 nm}).

Mar), and much of the APF/CBP was lost in the retentate of the 10-kd ultrafiltration step of this procedure, presumably because of self-aggregation of the protein.

Peaks 2 and/or 3 strongly bound calcium and were the most immunoreactive with PAb-APF-94, prepared against APF-

Mar peak 4, which did not bind calcium. Peaks 2 and 3 were the major polypeptide components of CBP-xol, the remainder consisted of pigment (peak 5).

A Proposed New Purification Procedure for APF/CBP

The calcium-binding functions of CBP-xol and CBP-new appeared to be little affected by extraction of lipids and pigments with organic solvents and fractionation by SDS-PAGE, but it is difficult to remove all the SDS. Together with the other findings, this suggests that the simplest procedure for isolation of CBP would involve: 1) precipitation of proteins from fresh hepatic bile by the addition of CaCl₂; 2) demineralization with alkaline EDTA; 3) removal of EDTA, calcium, and salts by dialysis or ultrafiltration through a 3.0-kd membrane; 4) extraction of the lyophilized retentate with *n*-butanol to remove lipids and pigments; 5) removal of butanol by washing with methanol and then lyophilization; 6) removal of large proteins by 30-kd ultrafiltration; 7) removal of small polypeptides and lipids from the step 6 filtrate by ultrafiltration through a hydrophobic 3.5-kd Omega membrane; and 8) separation of Ca⁺⁺- and lipid-binding components and residual pigment by hydrophobic HPLC. Whichever preliminary purification steps are used, preparative-scale versions of the Marseille hydrophobic HPLC system would seem the best final step to obtain truly pure preparations of APF (peak 4) and CBP (peaks 2 and 3).

The Duality of APF/CBP

Though limitations of the ⁴⁵Ca⁺⁺-binding method mean that the results cannot be considered precisely quantitative,^{1,12} they indicate that the APF/CBP preparations, extracted from gallstones or precipitated from bile by calcium, bind calcium strongly, whereas preparations derived from bile by serial ultrafiltration or density gradient ultracentrifugation show little interaction with calcium. The pigment/lipid and calcium-binding functions are clearly separated by hydrophobic HPLC (Figs. 3 and 4); thus, calcium-binding peaks 2 and 3 are most prominent in those APF/CBP preparations that bind calcium strongly, whereas peak 4, which did not bind calcium, dominates in preparations that show little cal-

TABLE 2. Amino Acid Analyses of APF/CBP Preparations

Source	Hepatic Bile			Mixed Stone		Xol Stone
Preparation	APF-Mar	CBP-New		CBP-Mix		CBP-xol
Amino Acid	<10 kd	12 kd	7 kd	12 kd	7 kd	12 kd
Tau	ND	ND	ND	0.0	0.0	ND
Hyp	0.0	0.0	0.0	0.0	0.0	0.0
Pro	3.3	4.5	4.9	4.9	5.4	5.2
Asp	10.0	10.1	9.3	13.4	13.0	12.5
Glu	12.3	12.9	14.6	13.7	14.0	14.8
Thr	6.1	5.1	5.1	5.5	4.7	6.6
Ser	7.9	9.1	8.7	7.2	7.2	7.6
Gly	19.8	11.2	11.6	12.5	14.7	14.0
Ala	7.1	7.1	7.4	8.4	8.4	6.0
Val	6.9	7.5	7.4	5.3	7.0	4.6
Ile	4.0	3.9	3.7	2.8	2.9	3.3
Leu	8.0	8.5	8.6	7.9	7.7	8.7
Cys/2	0.6	0.0	0.0	0.0	0.0	0.3
Met	0.0	1.9	1.9	0.1	0.0	0.0
Tyr	1.7	4.1	3.5	3.0	1.1	1.4
Phe	3.6	5.1	4.4	4.5	3.5	4.3
Trp	ND	ND	ND	ND	ND	ND
His	1.4	1.4	1.7	1.6	1.9	1.2
Lys	3.8	4.8	4.3	4.8	4.7	5.2
Arg	3.4	3.0	2.9	4.4	3.8	4.3
Total	100.0	100.0	100.0	100.0	100.0	100.0
mol% diacid	22.2	23.0	23.9	27.1	27.1	27.2
mol% dibasic	8.7	9.2	8.9	10.7	10.4	10.7
mol% Ser + Thr	14.0	14.2	13.8	12.8	<i>11.9</i>	14.2
diacid-dibasic	13.5	13.8	14.9	16.4	16.7	16.6

NOTE. With CBP-new 12 kd and 7 kd as reference: values in bold are significantly higher and values in italics are significantly lower. ND, not done in this analysis, but absence of Trp confirmed by independent, non-destructive analysis.

cium interaction. *CBP-xol* showed little of peak 1 and pure *CBP-Sea* lacked peak 5, yet both preparations strongly interacted with calcium, supporting the importance of HPLC peaks 2 and 3 in calcium interactions. Thus, all APF/CBP preparations appear to be mixtures of the two component proteins, lipid/pigment-binding APF and calcium-binding CBP, in different proportions. This contributes in part to the mutual cross-reaction of all preparations with polyclonal antibodies raised against any given preparation as antigen.

Despite their different mobilities on HPLC, the polypeptide backbones of APF and CBP may be nearly identical, because all APF/CBP preparations had similar amino acid compositions and they all reacted with MAb-32. Furthermore, the calcium-binding HPLC peaks 2 and 3 reacted avidly with PAb-APF-94, prepared against the more hydrophobic peak 4. Calcium-regulatory proteins in other tissues are known to exist in inactive forms, which do not bind calcium, but, upon phosphorylation or deamidation, they change their conformation to allow calcium binding.³⁷ We hypothesize that APF (HPLC peak 4) may correspond to the inactive form, which undergoes posttranslational modifications and conformational changes to yield the two calcium-binding CBP peaks 2 and 3. This hypothesis is compatible with both the more hydrophilic nature of peaks 2 and 3, and their greater immunoreactivity with PAb-APF-94. It is also possible that APF and CBP differ in their patterns and/or extent of glycosylation, reminiscent of the multiple glycosylated forms of pancreatic stone protein (lithostathine),³⁸ all of which react with the same monoclonal antibodies.³⁹ *CBP-pig*, prepared from gallstones, has been shown to be free of glycan groups,¹ but such an analysis has not been performed on APF preparations from bile.

Nature of the 7-kd and 12-kd Bands Seen on SDS-PAGE

The 7-kd and 12-kd bands of APF/CBP, regardless of the source or method of preparation, yielded amino acid patterns that were quite similar to each other (Table 2). On hydrophobic HPLC, each of the two bands of *CBP-xol* exhibited closely similar patterns (Fig. 4B and C). The 7-kd and 12-kd bands from every type of gallstone studied functioned similarly; both moieties inhibited the precipitation of calcium phosphate and bound to gallbladder mucin with similar affinity, forming complexes that altered the kinetics and structure of the formation of calcium phosphate.⁷

Though these findings suggested that the 12-kd band might be a dimer of the 7-kd band, each band eluted from a preparative gel ran true on second gel, with no evident dissociation of the 12-kd band to yield the 7-kd band, or association of the 7-kd band to yield the 12-kd band (Fig. 1). Thus, if the 12-kd band was a dimer, the two 7-kd bands had to be covalently linked. Both bands, however, were detected in the presence of mercaptoethanol, and each contained no cysteine and little methionine, excluding cross-linkage by S-S bond(s). The possibility that the 12-kd band consisted of two monomers linked by covalently bound bile pigment could not be confirmed because of the poor diazo-reactivity of the attached pigment. Although, in first-stage SDS-PAGE of Sephadex G-25 eluates, a higher quantity of pigment was associated with a greater proportion of the 7-kd band, purer preparations freed of most of the pigment by the Laemmli gel or diazotization yielded mainly the 7-kd band. We believe that the different electrophoretic migration of these otherwise similar proteins was caused by associated pigments and/or lipids.

Future Directions

Large amounts of CBP have been obtained recently, using the proposed new purification procedure, and fractionation by preparative hydrophobic HPLC is underway. Sequencing

of previous APF or CBP preparations has been unsuccessful thus far, despite the use of various techniques and strategies to unblock the N-terminus. Such approaches may prove more successful with the pure HPLC peaks. If not, attempts are planned to analyze these peaks by mass-spectroscopic determination of molecular weight and by C-terminal sequencing. Once the structure and functional domains of APF and CBP are known, they can be isolated by specific adsorption chromatography and/or synthesized by molecular biological techniques, allowing elucidation of their interrelationships and roles in gallstone formation.

REFERENCES

- Ostrow JD. APF/CBP, an anionic polypeptide in bile and gallstones that may regulate calcium salt and cholesterol precipitation from bile. *HEPATOLOGY* 1992;16:1493-1496.
- Ostrow JD. The roles of calcium salts and matrix in gallstone formation. In: Meyer zum Büschenfelde KH, Paumgartner G, Schölmerich J, eds. *Perspectives in Gastroenterology*. Munich: Urban and Schwarzenberg, 1995:143-152.
- Domingo N, Lafont H, Halpern Z, Peled Y, Grosclaude J, Gilat T. Anionic polypeptide fraction in bile of patients with and without gallstones. *HEPATOLOGY* 1993;17:778-780.
- Martigne M, Domingo N, Lafont H, Nalbhone G, Hauton JC. Purification of the human anionic polypeptide fraction of the bile lipoprotein complex by zonal ultracentrifugation. *Lipids* 1985;20:884-889.
- Nalbhone G, Lafont H, Vigne JL, Domingo N, Lairon D, Chabert C, Lechêne P, et al. The apoprotein fraction of the bile lipoprotein complex: isolation, partial characterization and phospholipid-binding properties. *Biochimie* 1985;61:1029-1041.
- Martigne M, Domingo N, Chanussot F, Nalbhone G, Lafont H, Hauton J. Effects of bile anionic polypeptide fraction on the fate of cholesterol carried by liposomes in the rat. *Proc Soc Exp Biol Med* 1988;187:229-234.
- Afdhal NH, Ostrow JD, Koehler RK, Niu N, Groen AK, Veis A, Nunes DP, et al. Interaction of bovine gallbladder mucin and calcium-binding protein: effects on calcium phosphate precipitation. *Gastroenterology* 1995;109:1661-1672.
- Shimizu S, Sabsay B, Veis A, Ostrow JD, Rege RV, Dawes LG. Isolation of an acidic protein from cholesterol gallstones, which inhibits the precipitation of calcium carbonate *in vitro*. *J Clin Invest* 1989;84:1990-1996.
- Okido M, Shimizu S, Ostrow JD, Nakayama F. Isolation of a calcium regulatory protein from black pigment gallstones: similarity with a protein from cholesterol gallstones. *HEPATOLOGY* 1992;15:1079-1085.
- Martigne M, Domingo N, De la Porte PL, Lafont H, Hauton J. Identification and localization of the apoprotein fraction of the bile lipoprotein complex in human gallstones. *Scand J Gastroenterol* 1988;23:731-737.
- Lechêne de la Porte P, Domingo N, van Wijland M, Groen AK, Ostrow JD, Lafont H. Distinct immuno-localization of mucin and other biliary proteins in human cholesterol gallstones. *J Hepatol* 1996;25:339-348.
- Kestell MF, Sekijima J, Lee SP, Park HZ, Long M, Kaler EW. A calcium-binding protein in bile and gallstones. *HEPATOLOGY* 1992;16:1315-1321.
- Domingo N, Grosclaude J, Bekaert ED, Mège D, Chapman MJ, Shimizu S, Ayralt-Jarrier M, et al. Epitope mapping of the human biliary amphipathic anionic polypeptide (APF): similarity with a calcium-binding protein (CBP) isolated from gallstones and bile and immunologic cross-reactivity with apolipoprotein A-I. *J Lipid Res* 1992;33:1419-1430.
- Schagger H, von Jagow G. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Anal Biochem* 1987;166:368-379.
- Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970;227:680-685.
- Suzuki H, Terada T. Removal of dodecyl sulfate from protein solution. *Anal Biochem* 1988;172:259-263.
- DeMoreno MR, Smith JF, Smith RV. Silver staining of proteins in polyacrylamide gels: increased sensitivity through combined Coomassie blue-silver stain procedure. *Anal Biochem* 1985;151:466-470.
- Chicz RM, Regnier FE. High-performance liquid chromatography: effective protein purification by various chromatographic modes. *Methods Enzymol* 1990;182:392-421.
- Trotman BW, Rajagopalan C, Bernstein SE. Monoconjugated bilirubin is a major component of hemolysis-induced gallstones in mice. *HEPATOLOGY* 1988;8:919-924.
- Blancaert N, Heirwegh KPM. Analysis and preparation of bilirubins and biliverdins. In: Ostrow JD, ed. *Bile Pigments and Jaundice: Molecular, Metabolic and Medical Aspects*. New York: Dekker, 1986:31-79.
- Penke A, Ferenczi R, Kovács K. A new acid hydrolysis method for determining tryptophan in peptides and proteins. *Anal Biochem* 1974;60:45-50.
- Gurantz D, Laker MF, Hofmann AF. Enzymatic measurement of choline-containing phospholipids in bile. *J Lipid Res* 1981;22:373-376.
- Mashige F, Kazuhiro I, Osuga T. A simple and sensitive assay of total serum bile acids. *Clin Chim Acta* 1976;70:79-86.

24. Kates M. Isolation, analysis and identification of lipids. In: Work TS, Work E, eds. *Techniques in Lipidology (Laboratory Techniques in Biochemistry and Molecular Biology, vol. 3, part 2)*. Amsterdam: Elsevier-North Holland, 1972:269-610.
25. Duck CG, Hong C, Baker CJ. Rapid quantitative analysis of polar and non-polar lipids in a single sample using three-way thin-layer chromatography. *Lipids* 1983;18:387-389.
26. Rendell M, Kao G, Mecherikunnel P, Petersen B, Duhaney R, Nierenberg J, Rasbold K, et al. Aminophenylboronic acid affinity chromatography and thiobarbituric acid colorimetry compared for measuring glycated albumin. *Clin Chem* 1985;31:229-234.
27. Mukerjee P. Use of ionic dyes in the analysis of ionic surfactants and other ionic organic compounds. *Anal Chem* 1956;28:870-873.
28. Thorin B, Gache G, Dubois T, Grataroli R, Domingo N, Russo-Marie F, Lafont H. Annexin VI is secreted in human bile. *Biochem Biophys Res Commun* 1995;209:1039-1045.
29. Ostrow JD, Mukerjee P, Tiribelli C. Structure and binding of unconjugated bilirubin: relevance for physiological and pathophysiological function. *J Lipid Res* 1994;35:1715-1737.
30. Fevery J, Blanckaert N, Leroy P, Michiels R, Heirwegh KPM. Analysis of bilirubins in biological fluids by extraction and thin-layer chromatography of the intact tetrapyrroles: application to bile of patients with Gilbert's syndrome, hemolysis, or cholelithiasis. *HEPATOLOGY* 1983;3:177-183.
31. Gautam A, Seligson H, Gordon ER, Seligson D, Boyer JL. Irreversible binding of conjugated bilirubin to albumin in cholestatic rats. *J Clin Invest* 1984;73:873-877.
32. Yoshida H, Inagaki T, Hirano M, Sugimoto T. Analyses of azopigments obtained from the delta fraction of bilirubin from mammalian plasma (mammalian biliprotein). *Biochem J* 1987;248:179-184.
33. Ohkubo H, Ostrow JD, Carr SH, Rege RV. Polymer networks in pigment and cholesterol gallstones assessed by equilibrium swelling and infrared spectroscopy. *Gastroenterology* 1984;87:805-814.
34. Lucassen J. The diazo reaction of bilirubin and bilirubin diglucuronide [PhD Thesis, University of Utrecht]. Utrecht: Kemink en Zoon, 1961.
35. Moore EW. The role of calcium in the pathogenesis of gallstones: Ca^{2+} electrode studies of model bile salt solutions and other biologic systems. *HEPATOLOGY* 1984;4:228S-243S.
36. Shimizu S, Izumi Y, Yamazaki M, Shimizu K, Yamaguchi T, Nakajima H. Anti-bilirubin monoclonal antibody. I. Preparation and properties of monoclonal antibodies to covalently-coupled bilirubin-albumin. *Biochim Biophys Acta* 1998;967:255-260.
37. Veis A, Sabsay B. Bone and tooth formation: insights into biomineralization strategies. In: Westbroek P, Dejon EW, eds. *Biomaterialization and Biological Metal Accumulation: Biological and Geological*. Dordrecht, the Netherlands: D. Riedel, 1982:273-284.
38. DeCaro A, Bonicel JJ, Rouimi P, DeCaro JC, Sarles H, Rovey M. Complete amino acid sequence of an immunoreactive form of human pancreatic stone protein. *Eur J Biochem* 1987;168:201-207.
39. Montalto G, Lusher M, DeCaro A, Multigner L, Sarles H, Delaage M. Monoclonal antibody analysis of human pancreatic juice components related to the pancreatic calculi protein. *C R Acad Sci (Ser C)* 1985;300:199-202.