Purification and characterization of a cholesterol-binding protein from human pancreas

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The protein composition of human intestinal lavage fluids was analysed by electroimmunoassay. In addition to secretory immunoglobulin A and other components that were antigenically related to serum proteins, a number of gut-specific proteins were detected. One of these was found to exhibit the capacity of binding sodium deoxycholate and cholesterol. After isolation of this cholesterol-binding protein from intestinal fluids, immunohistochemical studies utilizing a specific antiserum indicated the pancreas to be the organ of its synthesis. The protein was subsequently purified from necrobiotic pancreas tissues and was found to be composed of a single polypeptide chain with a mol.wt. of 28 000 and an isoelectric point of pH 4.9. The deoxycholate-binding capacity determined by gel chromatography in the presence of [^3H]deoxycholate was calculated to be approx. 24 mol of deoxycholate/mol of protein. In the intestinal fluids the protein appeared to be present in firm association with cholesterol, phospholipids, triacylglycerols and bile salts as a macromolecular protein–lipid complex. The possibility is raised that the pancreas-derived, cholesterol-binding protein may fulfil a function as an intestinal ‘lipoprotein’.

Intestinal lavage before abdominal surgery or diagnostic X-ray procedures permits the recovery of proteins that are secreted into the gut in a form suitable for bio-immunochemical analyses. Lavage is performed by introducing 5–6 litres of saline (0.9% NaCl) via an intragastric tube into the stomach over a period of 2–4 h. Faecal components excreted within the first 3–4 litres of the lavage fluid are discarded. The residual 1–2 litres of lavage solution recoverable thereafter contain gut proteins that are secreted from the mucus membranes, the bile and the pancreas. Contaminations with bacteria and cellular debris are thereby reduced to a minimum.

In the present paper we report on the composition of proteins present in such intestinal-lavage solutions. Electroimmunoassays led to the detection of a hitherto-undescribed gut-specific protein that exhibited the capacity to bind deoxycholate and cholesterol. We first describe the detection and isolation of this cholesterol-binding protein from lavage fluids. Thereafter we report on the identification of the pancreas as the organ of its synthesis, the purification of the native protein from necrobiotic pancreas tissues, and its biochemical charac-

terization. The possibility is raised that the protein may represent a special intestinal ‘lipoprotein’ that plays a role in intestinal transport and metabolism of cholesterol.

Experimental procedures

Chemicals

Agarose type HSA was obtained from Litex, Glostrup, Denmark; Sephadex G-75, Protein-A–Sephadex, DEAE-Sephadex CL-6B and protein kit MS II from Pharmacia; Servalyt Ampholines pH 2–11, acrylamide, bisacrylamide, *NNN'*-tetramethylethylenediamine, Coomassie Brilliant Blue R-250, cholesterol, deoxycholate, SDS, and calibration proteins for molecular-weight determination by gel-filtration were from Serva; pH 3.5–10 Ampholine was from LKB; dithiothreitol, trypsin, chymotrypsin and *N*-acetyl-*NNN'*-trimethylammonium bromide were from Merck, Darmstadt, Germany; Freund's incomplete adjuvant was from Difco; PM 10 membranes for ultrafiltration were from Amicon Corporation; [$4\text{-}^{14}\text{C}$]cholesterol, [^3H]cholesterol, [$4\text{-}^{14}\text{C}$]progesterone, [$1\text{-}^{14}\text{C}$]glycocholic acid, cholesteryl [$1\text{-}^{14}\text{C}$]oleate and tri[$1\text{-}^{14}\text{C}$]palmitoylglycerol were from Amersham Buchler, Braunschweig, Germany; [^3H]deoxycholic acid was pur-

Abbreviations used: SDS, sodium dodecyl sulphate; Ig, immunoglobulin.

chased from New England Nuclear Corp. Kodak Definix medical films and LKB Ultrafilm were used for autoradiography. Rabbit antibodies against human serum proteins, and specific antisera against single serum proteins, were purchased from DAKO patts, Copenhagen, Denmark, and Behringwerke, Marburg, Germany. All other chemicals were reagent or analytical grade.

Materials

Intestinal lavage fluids. Lavage fluids were obtained with informed consent from patients suffering from intestinal diseases. The last 1–2 litres of opaque, yellowish fluid were centrifuged (Sorvall RC 2-B; GSA rotor; 12000 rev./min 20 min; 4°C) and the supernatant concentrated 50–100-fold by pressure dialysis (Amicon PM 10 membranes). Concentrated lavage fluids from several patients were pooled and used as antigen for immunization of rabbits.

Pancreas tissues. Necrobiotic pancreas tissues were supplied by the Pathological Institute at the University of Giessen. The organs were removed during routine sections and stored at –20°C. Batches of 15–18 organs were employed for each preparation procedure.

Antisera. Antisera were raised in rabbits by using the immunization procedure described by Harboe & Ingild, (1973). After the second booster injection, rabbits were repeatedly bled by puncture of the marginal ear vein.

Methods

Electroimmunoassay. Rocket-fused rocket-crossed and charge-shift-crossed immunoelectrophoresis were performed in 1% (w/v) agarose gels as described by Axelsen *et al.* (1973) and Bhakdi *et al.* (1977).

Autoradiographic analyses were employed to investigate the specific binding of various ligands to proteins. To demonstrate the binding of cholesterol, 5 μ Ci of 14 C-labelled cholesterol were dried under N_2 to a thin film on glass. Samples (50 μ l) were added and incubated for 1 h at 37°C. Thereafter crossed immunoelectrophoresis was performed. The plates were washed, dried and covered with an X-ray film, which was developed after 4 days of exposure. In an identical procedure, cholesteryl [14 C]oleate, [14 C]-progesterone and tri[14 C]palmitoylglycerol were used.

Preparative agarose-gel electrophoresis. Preparative agarose-gel electrophoresis was performed in 1% (w/v) agarose in an isoelectric-focusing tray (LKB) (10.8 cm $\times$ 24.1 cm $\times$ 0.5 cm). Agarose and buffer were supplemented with 0.2% deoxycholate. A 1 cm strip was removed at one end and filled with the crude protein solution mixed with an equal volume of 1% (w/v) agarose. Electrophoresis was performed at 10 V/cm for about 3 h at 8–10°C. The

positions of the proteins were determined by electrophoresing a small axial strip against polyspecific antiserum. The agarose gels were sectioned in 1 cm strips and proteins were eluted electrophoretically in 50 mM-glycine/20 mM-Tris, pH 8.7, at 320 V for 18 h.

SDS/polyacrylamide-gel electrophoresis

Discontinuous slab gels (1.5 mm thickness), prepared as described by Bjerrum & Bhakdi (1977) and electrophoresed by the Laemmli (1970) procedure were used to monitor the purification procedures and for molecular-weight determination. Calibrations were made with standard proteins from Pharmacia (phosphorylase *b*, mol.wt. 94 000; bovine serum albumin, 67 000; ovalbumin, 43 000; carbonic anhydrase, 30 000; soya-bean trypsin inhibitor, 20 100; and lactalbumin, 14 400).

Isoelectric focusing. Polyacrylamide isoelectric-focusing gels were prepared as described by Bhakdi *et al.* (1980). Ampholines (1%, pH 3.5–10 and pH 4–6 from LKB as well as Servalyt pH 2–11) were used. Focusing was carried out at 220 V for 18 h at 4°C. Gels were sectioned into 2 mm slices and eluted for 2 h in water. Thereafter the pH was determined and the protein identified by electrophoresis of the gel sections and also of unsectioned gels against specific antiserum. Gels were stained for carbohydrate with Schiff's reagent (Fairbanks *et al.*, 1971).

Amino acid analysis. Samples were hydrolysed in evacuated sealed tubes with 6 M-HCl at 110°C for 24 h.

Protein determination. Protein was determined as described by Lowry *et al.* (1951) and immunochemically by rocket immunoelectrophoresis (Laurell, 1972), a standard concentration of specific antiserum and antigen being used.

Thin-layer chromatography. T.l.c. was carried out on pre-coated silica-gel plates (Merck). Samples (15 μ l) were applied under a stream of air and the chromatography was performed in chloroform/methanol/water (65:25:4, by vol.) as solvent. After drying, plates were sprayed with ethanolic 0.05 M- H_2SO_4 and developed by heating (Rouser *et al.*, 1967).

Determination of cholesterol. The quantitative determination of cholesterol was performed enzymically by a commercially available test combination purchased from Boehringer Mannheim and by the Liebermann–Burchard procedure (Merck, 1974).

Immunohistology. The unlabelled antibody enzyme method was used (Sternberger *et al.*, 1970). Briefly, paraffin-embedded sections (5 μ m thick) of pancreas tissue were first incubated with 0.5% H_2O_2 in methanol for 30 min at 37°C to eliminate endogenous peroxidases. After washing, sections were incubated with the following solutions in

sequence: normal pig serum diluted 1:5 in saline (0.9% NaCl) for 30 min at 37°C; specific rabbit antibodies to the cholesterol-binding protein for 1 h at 37°C and for 12 h at 4°C; normal pig serum (diluted 1:10) to minimize non-specific absorption; pig antibodies against rabbit immunoglobulins (diluted 1:10) for 1 h at 37°C; horseradish peroxidase-anti-peroxidase complex from rabbits, diluted 1:50 in saline, for 1 h at 37°C. Fixation of the peroxidase was detected by a chromogen in the presence of H_2O_2 (Hanker *et al.*, 1977) and sections were counterstained by Haemalaun. As a control, sections were incubated with antiserum against insulin, staining selectively β -cells of the islets.

Isolation of immunoglobulins from antiserum. The immunoglobulin fraction was prepared by adsorption on a Protein A-Sepharose CL-4B column (Pharmacia) equilibrated with 38 mM-Tris/100 mM-glycine/15 mM- NaN_3 , pH 8.6, at 4°C; 2 vol. of serum were applied to 1 vol. of gel. Thereafter the column was washed with 2 vol. of Tris/glycine, 1.5 vol. of 0.5 M-NaCl/100 mM- $NaHCO_3$, pH 8.3, and finally 1 vol. of Tris/glycine. The immunoglobulins were then eluted by 1.5 vol. of 1 M-acetic acid, the eluate being buffered with 1 vol. of Tris/glycine and dialysed against 10 mM-Tris/50 mM-NaCl/15 mM- NaN_3 , pH 8.2. Fixation as well as elution of the immunoglobulins was tested by electroimmunoassays.

Isolation of the cholesterol-binding protein from human pancreas tissues. Batches of 15–18 organs from necrobiopsies were macroscopically freed from adjacent and fatty tissues. The organs were placed in 2 litres of 10 mM-Tris/HCl, pH 6.0, cooled on ice, and homogenized in a commercial Waring Blender for 5 min. Thereafter the tissue homogenate was centrifuged in a Sorvall RC-3 centrifuge (rotor HG-4) at 7000g for 30 min at 0°C. Three layers developed, of which the intermediate layer was recovered and extracted with 8% (v/v) Triton X-100 for 3 h at 22°C with continuous stirring. During this time the tissue solution became dark-coloured and opaque. An equal amount of sucrose (w/v) was added to the homogenate and the viscous fluid poured into a separatory funnel. Over a 48 h period at room temperature, virtually all lipids floated to form a sharply separated, turbid top layer constituting approx. one-quarter of the total volume. The bulk of cholesterol-binding protein was recovered in the clear lower part and was stored at 4°C. Portions (250 ml) were dialysed against 6 litres of 10 mM-Tris/HCl, pH 6.0, for 2 days with one change of buffer and then applied to a 265 ml column of DEAE-Sepharose Cl-6B (Pharmacia) equilibrated with the same buffer. After application of the material, protein was eluted with 1 litre of a linear 100–600 mM-NaCl gradient in the same Tris/HCl

buffer, pH 6.0. The column was eluted at a flow rate of 44 ml/h and fractions (22 ml) were collected. The eluted fractions were analysed by fused-rocket immunoelectrophoresis with the use of specific as well as polyspecific antibodies, and by SDS/polyacrylamide-gel electrophoresis. Fractions containing the cholesterol-binding protein were pooled, concentrated by pressure dialysis to 5–8 ml, and applied to a Sephadex G-75 column. The cholesterol-binding protein isolated by this procedure was analysed by crossed immunoelectrophoresis and SDS/polyacrylamide-gel electrophoresis. An average of 1 mg of protein was obtained per organ. The concentration of the cholesterol-binding protein in wet pancreas tissue was roughly estimated by rocket electrophoresis to be 8–17 mg/100 g. The recovery after purification was calculated to be in the order of 4–8%.

Results

Immunoelectrophoretic analyses

Crossed immunoelectrophoresis of concentrated intestinal lavage fluids developed with antibodies to human serum proteins yielded a pattern that was fairly consistent among different individuals (Fig. 1). By using monospecific antisera in intermediate gels, the major immunoprecipitates were identified as secretory IgA, antichymotrypsin, α_1 -antitrypsin and acid α_1 -glycoprotein. Transthyretin, the third complement component (C3), IgG and IgM constituted minor components, whereas albumin was absent in most cases.

Since the above experiments permitted only the detection of those components that were antigenically related to serum proteins, further analyses were performed with the use of an antiserum that had been developed against the lavage proteins. In the immunoelectrophoresis shown in Fig. 2, antibodies to serum proteins were incorporated into a first (intermediate) gel, and antiserum to lavage proteins into the second (upper) gel. Gut-specific proteins not reacting with the former antibodies were thus precipitated and identified in the upper gels. In this manner, three to five gut-specific proteins could be defined (Fig. 2). The immunoprecipitation pattern of the gut-specific proteins varied among individual donors. Attention was, however, drawn to one protein moiety (arrow), which was present in all lavage fluids tested but which gave rise to immunoprecipitates of non-uniform morphology. At this stage, the possibility was considered that the immunoprecipitate might derive from a protein-ligand complex of varying size and/or charge. Additional experiments using charge-shift electrophoresis lent support to this contention. This technique permitted differentiation of proteins that bound detergent from those that did not. Amphi-

Fig. 1. Two-dimensional immunoelectrophoresis of concentrated lavage fluids against a serum protein antiserum. First-dimension electrophoresis was performed for 45 min at 10 V/cm. Second-dimension immunoelectrophoresis was performed at 2 V/cm for 16 h in gels containing antibodies to human serum proteins ($12 \mu\text{l}/\text{cm}^2$; DAKO, code 100 SF) (I). The intermediate gel in the left immunoplate (a) was blank; the right intermediate gel contained antiserum against α_1 -antitrypsin ($2 \mu\text{l}/\text{cm}^2$; DAKO) (b) as an example for identification of the proteins 1–7: 1, secretory IgA; 2, IgM; 3, complement component C3; 4, α_1 -antitrypsin; 5, acid α_1 -glycoprotein; 6, antichymotrypsin; 7, transthyretin.

Fig. 2. Two-dimensional immunoelectrophoresis of three lavage fluids (a–c) against two antisera. I, antiserum to serum proteins; II, antiserum against lavage-fluid proteins; A, secretory IgA. The arrows point to a gut-specific protein exhibiting varying electrophoretic mobility.

philic proteins that bind Triton X-100 exhibit bidirectional 'charge-shifts' in deoxycholate and *N*-acetyl-*NNN*-trimethylammonium bromide; those that bind deoxycholate or *N*-acetyl-*NNN*-trimethylammonium bromide only exhibit unidirectional charge shifts in the respective detergent, whereas those that bind no detergent exhibit no changes in electrophoretic mobility in any one buffer/detergent system.

Fig. 3 illustrates the unidirectional charge-shift of the depicted gut-specific protein (arrow) in the presence of deoxycholate. In this experiment, lavage proteins were first chromatographed over Sephacryl S-300 to effect partial purification of the protein. Fractions containing the protein were concentrated and analysed by charge-shift crossed immunoelectrophoresis. The very selective effect of deoxy-

cholate on the electrophoretic mobility of the gut-specific protein is apparent; other protein moieties were unaffected. No charge-shift of the protein was found in the presence of *N*-acetyl-*NNN*-trimethylammonium bromide and the immunoprecipitation pattern of the proteins in this detergent-buffer system was identical with that of Fig. 3(a), developed in the presence of Triton as the sole detergent (immunoplate not shown). Thus, charge-shift electrophoresis was indicative of selective binding of deoxycholate by the lavage protein under study.

Evidence that the sterol structure is responsible for deoxycholate-binding

The binding of deoxycholate could be due to the

Fig. 3. Charge-shift immunoelectrophoresis of partially purified lavage fluid against antiserum I and II (Fig. 2) First-dimension electrophoresis was performed in gels containing Triton X-100 (a) and Triton X-100 + deoxycholate (b) for 4 h at 4 V/cm. The arrow points to the gut-specific protein showing a deoxycholate-induced charge shift. When the electrophoresis was run in the presence of Triton X-100 and *N*-acetyl-*NNN*-trimethylammonium bromide, results were identical with those in (a).

Fig. 4. Autoradiography of the crossed immunoelectrophoresis of human serum (a) and lavage fluid (b) after incubation with [^{14}C]cholesterol

The radioactive smear in the lower part of (b) is an artefact deposit of cholesterol occurring during second-dimension electrophoresis and was not seen in autoradiograms with purified cholesterol-binding protein from human pancreas. Antiserum I and II are as described in Fig. 2. α -LP, α -lipoprotein; β -LP, β -lipoprotein; HS, human serum; LF, lavage fluid.

negative charge of the bile salt or to the sterol structure of the molecule. To differentiate between these possibilities, the binding of various radiolabelled sterols was tested by autoradiography subsequent to electroimmunoassay. This technique permitted recognition of proteins that combined with the radiolabelled ligand under investigation. An experiment wherein whole human serum was pre-incubated with [^{14}C]cholesterol and subjected to crossed immunoelectrophoresis is shown in Fig. 4(a). Only the immunoprecipitates corresponding to apo-A- and apo-B-containing serum lipoproteins caused a blackening of the film. When a parallel experiment was performed with lavage proteins, one

immunoprecipitate selectively caused blackening of the film, and this corresponded to the above described enteral protein which exhibited the charge shift in deoxycholate. (Fig. 4b). The blackening smear in the lower gel is an artefact, possibly arising from partial dissociation of cholesterol from the protein during the electroimmunoassay. Analogous experiments were also performed with [^{14}C]glycocholic acid, [^{14}C]cholesteryl oleate, [^{14}C]progesterone and [^{14}C]tripalmitoylglycerol as ligands. Glycocholic acid and the cholesteryl ester were found to bind to the same enteric protein, whereas the triacylglycerol and progesterone did not (results not shown).

Isolation of the cholesterol-binding protein from intestinal lavage fluids

A typical chromatographic separation of lavage fluid proteins over Sephadex G-75 is depicted in Fig. 5(a). The eluting positions of the proteins were determined by fused-rocket immunoelectrophoresis developed with antibodies to human serum proteins (I) and to total lavage fluids (II). Fig. 5(b) depicts a rocket immunoelectrophoresis of the same fractions developed with a specific antiserum to the cholesterol-binding protein. The coalescence of the rocket immunoprecipitates demonstrates the antigenic identity of the protein moieties that eluted as two peaks (arrows, Fig. 5a) on the column. Thus the protein exists in two molecular forms that differ in size from one another. Cholesterol determinations revealed that the sterol eluted as a peak in the same fractions as the first protein peak. By contrast, the rear protein peak was virtually lipid-free (see the Table in the legend to Fig. 5). These latter fractions were pooled and subjected to preparative agarose-gel electrophoresis. Gel sections containing the protein were eluted and the proteins re-chromatographed to a Sephadex G-75 column. This step yielded the cholesterol-binding protein in immunologically pure form, as determined by crossed immunoelectrophoresis (Fig. 6).

On SDS/polyacrylamide-gel electrophoresis, one polypeptide band of mol.wt. 28 000 was found. Occasionally a weaker band corresponding to 26 000 mol.wt. was also observed that might represent a contaminant or degradation product of the cholesterol-binding protein. Both protein bands were unaffected by the incubation with 20 mm-dithiothreitol and were negative in the periodate/Schiff reaction.

Identification of the pancreas as the organ of synthesis of the cholesterol-binding protein

A specific antiserum was raised against the purified protein. Electroimmunoassays performed with this antiserum were used to search for the presence of the protein in diverse body fluids including serum, sputum, urine, lachrymal fluid, semen, bile and pancreatic secretions. The protein was found exclusively in pancreatic juice. Subsequently, immunohistological studies were performed on sections of paraffin-embedded pancreas tissues. The cytoplasm of the exocrine glands stained selectively with the antibodies, indicating these to be the sites of production of the protein. It was not possible to define the cell type within the exocrine glands that are involved in the synthesis of the cholesterol-binding protein. A series of sections

Fig. 5. Quantification of cholesterol-binding protein from human pancreas (CBPP) and cholesterol (Chol.) in the Sephadex G-75 column fractions of concentrated lavage fluids

(a) Fused-rocket immunoelectrophoresis of fractions 14–30 against antiserum I and II (described in Fig. 2). (b) Rocket immunoelectrophoresis against specific CBPP antiserum (10 µl/cm²). Calibrations with purified CBPP (10, 20 and 30 µg/ml).

		Concn. (μg/ml)													
Fraction	...	14	15	16	17	18	19	20	21	22	23	24	25	26	
CBPP		8	28	32	30	22	9	5	5	6	12	18	16	10	
Chol.		92	312	351	338	181	32	—	—	—	—	—	—	—

Fig. 6. Crossed immunoelectrophoresis of purified cholesterol-binding protein from human pancreas (2.5 μg) against antiserum I and II
First dimension: 10 V/cm, 45 min; second dimension: 2 V/cm, 14 h.

prepared from pancreas tissues definitively indicated that the islets do not react with antibodies against the cholesterol-binding protein.

Isolation of cholesterol-binding protein from human pancreas

The cholesterol-binding protein was isolated from necrobiotic pancreas tissues by using the procedure detailed under 'Methods'. The considerable difficulties were initially encountered in our endeavours to remove the bulk of tissue debris and lipids. Empirically, it was found that solubilization of tissue homogenates with Triton X-100, followed by lipid flotation at 1 g in sucrose led to effective delipidation of the proteins, which could then be fractionated by ion-exchange chromatography. Fig. 7 depicts the result of such a chromatography; the cholesterol-binding protein was detected with the use of the specific antiserum and SDS/polyacrylamide-gel electrophoresis performed in parallel. A single subsequent chromatography over Sephadex G-75 led to recovery of the protein in satisfactorily purified form.

Biochemical characterization of the cholesterol-binding protein

The molecular radius of the protein was determined to be 2.3 nm by gel filtration. The sedimentation coefficient ($s_{20,w}$) was 2.8S as determined by

Fig. 7. DEAE-cellulose column chromatography of delipidated pancreas tissue homogenate equilibrated with 0.01 M-Tris/HCl, pH 6.0
Proteins were eluted with a 100–400 mM-NaCl gradient. Fused-rocket immunoelectrophoresis (a) and SDS/polyacrylamide-gel electrophoresis (b) of fractions 8–44 are depicted. The NaCl concentration is shown in (c). Abbreviations used: CBPP, cholesterol-binding protein from human pancreas; HSP, human serum protein.

Table 1. Amino acid composition of the cholesterol-binding protein

All data including the amount of glucosamine were estimated from the 6M-HCl/110°C/24 h hydrolysate. No correction was made for serine and threonine.

Amino acid	Content (mol/mol of protein)	Amino acid	Content (mol/mol of protein)
Lys	8.2	Ala	17.6
His	5.1	½Cys	9.4
Arg	9.7	Val	17.6
Asp	30.2	Met	1.4
Thr	14.9	Ile	10.7
Ser	27.7	Leu	19.1
Glu	19.3	Tyr	9.6
Pro	18.9	Phe	6.5
Gly	31.0	Glucosamine	2.9

Fig. 8. Determination of the deoxycholate binding capacity of cholesterol-binding protein from human pancreas (CBPP). Gel filtration of CBPP in the presence of 10 mM-deoxycholate/[^3H]deoxycholate. The column contained Sephacryl S-200 and was 0.9 cm $\times$ 58 cm. The fractions were assayed for CBPP (●) and [^3H]deoxycholate (○). V_0 and V_t denote the void elution volume and the column bed volume respectively.

analytical centrifugation. A partial specific volume of 0.72 was calculated from the amino acid composition, and from these values a mol.wt. of 27000–28000 was calculated. This was in close agreement with mol.wt. 28000 determined by SDS/polyacrylamide-gel electrophoresis.

The amino acid composition of the cholesterol-binding protein is given in Table 1. The isoelectric point as determined by isoelectric focusing was pH 4.9.

Quantitative deoxycholate binding studies were performed by chromatographing the purified protein over a Sephacryl S-200 column equilibrated with radioactive detergent. These experiments indicated that binding of deoxycholate occurred only above the critical micelle concentration of the detergent; when deoxycholate was present in submicellar concentrations (1.25 mM), no elevation of radioactivity over the baseline value was observed in the protein-containing fractions. In this system, the protein eluted at a position corresponding to a 20000–30000-mol.wt. protein. When the deoxycholate concentration was raised above the micellar concentration (10 mM), radioactivity was elevated above the baseline value, indicating co-elution of bound detergent with the protein, and the eluting position of the protein–detergent complex shifted to a region corresponding to that of a 30000–40000-mol.wt. protein. The amount of protein-bound detergent was determined by correlating the increase of radioactivity with the protein concentration in the corresponding fractions and was calculated to be

approx. 24 molecules of deoxycholate per molecule of cholesterol-binding protein (Fig. 8).

Discussion

The immunochemical analyses of intestinal lavage fluids showed that relatively few serum proteins were excreted via the intestine. Regularly, a high content of secretory IgA, α_1 -antitrypsin and antichymotrypsin were found, whereas albumin was generally absent or present only in minor quantities. As expected, only traces of IgG and IgM were detectable (Tomasi & Grey, 1972). Somewhat surprisingly, the first complement component and serum lipoproteins, reportedly synthesized in the mucosa of the gut (Colten *et al.*, 1968; Osborne & Brewer, 1977), were never found, whereas complement component C3 apparently represents a normal minor constituent of the lavage fluids. At present, it is not possible to correlate the various immunoelectrophoresis patterns obtained with any defined gastrointestinal diseases.

With the availability of antisera against lavage-fluid proteins, gut-specific proteins were discovered in addition to the serum-related proteins. One of these proteins attracted attention by its ability to bind deoxycholate, as indicated by charge-shift crossed immunoelectrophoresis. Autoradiographical analyses of ligand binding in crossed immunoelectrophoresis subsequently showed that cholesterol and cholesteryl esters also bound to the protein, whereas progesterone and triacylglycerols

did not. These preliminary studies indicate that the binding capacity of the cholesterol-binding protein seems to be restricted to steroids, and that the side chain at C-17 or the substituent at C-3 may influence the protein-steroid interaction.

In the lavage fluids, the cholesterol-binding protein appeared to be present in two different molecular forms. On gel chromatography, high-molecular-weight material co-eluted with cholesterol, phospholipids and many other proteins, whereas lipid-free protein was recovered in low-molecular-weight fractions. We succeeded only in the purification of the low-molecular-weight protein. The procedure was cumbersome and yields varied greatly, depending on the distribution of the protein in the chromatography fractions. Attempts to purify the protein from high-molecular-weight fractions uniformly failed. In particular, attempts to initially delipidate the protein were unsuccessful. For example, adsorption of the protein-lipid complex to DEAE-Sepharose followed by extensive washings with 0.5% Triton X-100, by analogy to the delipidation of membrane proteins (Simons *et al.*, 1973), the treatment with chloroform/methanol (Lux *et al.*, 1972) and the ether extraction (Dean *et al.*, 1967), did not result in quantitative delipidation of the protein. The use of the organic solvents, on the other hand, was accompanied by a partial denaturation of the protein (A. Sziegoleit, unpublished work).

Since immunohistological studies supplied evidence that the protein is synthesized in the pancreas, a procedure was then developed to isolate the protein from necrobiotic pancreases. For this purpose, batches of pancreases were homogenized and, in a first step, lipid was removed by flotation in a Triton/sucrose solution. The given concentrations of Triton and sucrose were elaborated in a series of experiments from which it became clear that flotation of lipids was dependent on the concentration of both detergent and sucrose. The simple and mild method for tissue delipidation may prove to be generally useful for isolation of other cytoplasmic proteins.

The protein isolated from pancreas tissues was antigenically identical with that derived from the intestinal lavage fluids. On SDS/polyacrylamide-gel electrophoresis the pancreas-derived protein yielded a single major polypeptide band of apparent mol.wt. 28000, a value that agreed with the molecular weight determined by gel chromatography and with the sedimentation coefficient of 2.8S found by analytical ultracentrifugation. The isolated protein was found to be very stable towards the action of proteinases, and was not degraded by trypsin or chymotrypsin, a property that would render it well suited to the enteric milieu.

We were unable to quantify the binding of

cholesterol to the protein *in vitro*, owing to the insolubility of cholesterol in water. Therefore quantitative ligand-binding studies were confined to deoxycholate, and these indicated that approx. 24 molecules of deoxycholate associated with one protein molecule *in vitro* to yield protein-detergent complexes of mol.wt. 36000. *In vivo*, the protein may, however, form a complex not only with cholesterol and bile acids, but also with phospholipids and triacylglycerols. The nature of the interactions *in vivo* is presently not understood and requires further investigation.

Although exocrine pancreatic proteins have been extensively studied, the cholesterol-binding protein described here has apparently not been detected previously. Methods employed in previous investigations included electrophoresis of unfractionated pancreatic secretions on cellulose acetate (Robinson *et al.*, 1970), analysis of pancreatic proteins by polyacrylamide disc electrophoresis (Keller & Allan, 1966; Allan *et al.*, 1970), immunoprecipitation (Clemente *et al.*, 1972), and, more recently, two-dimensional separations by isoelectric focusing linked to SDS/polyacrylamide-gel electrophoresis (Scheele *et al.*, 1981). The cited studies have led to a description of 19 defined exocrine pancreatic proteins. Of these, the biochemical properties of trypsin and chymotrypsin are most closely related to those of the cholesterol-binding protein. Therefore we tested whether the isolated protein was capable of cleaving synthetic substrates of these classical proteinases. Neither tosyl-L-arginine methyl ester nor *N*-benzoyl-L-tyrosine ethyl ester was cleaved, excluding a similarity between the protein and these proteinases.

The cholesterol-binding protein appears to represent a very minor component of pancreatic secretions. There have been reports of cholesterol-binding proteins in the liver (Ritter & Dempsey, 1973; Bloj & Zilversmit, 1977; Erickson *et al.*, 1978) and adrenals (Lefevre *et al.*, 1978), but as yet there is no knowledge of a cholesterol-binding protein in the enteric secretions, although the existence of such a protein has been suspected (Gangl & Ockner, 1975). In the proximal bowel, dietary cholesterol is mixed with cholesterol secreted with the bile and forms mixed micelles with phospholipids and bile acids. During the intestinal passage approx. 98% of the bile acids are resorbed, whereas resorption of cholesterol is in the order of 50%. This results in a decrease in the bile acids/cholesterol molar ratio to about 1 in the faeces (Dietschy & Wilson, 1970; Angelin, 1977), which is incompatible with a micellar solubilization of cholesterol (Dam & Hegardt, 1971; La-Russo *et al.*, 1975). It is possible that the pancreatic cholesterol-binding protein acts to balance the interaction of bile salts and cholesterol during the course of their intestinal passage, preventing the

aggregation of the insoluble sterol at these low concentrations of bile acids. The protein may thus represent a hitherto unrecognized component in the intestinal metabolic pathway of cholesterol.

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