Subcellular Distribution of Bile Acids, Bile Salts, and Taurocholate Binding Sites in Rat Liver†

F. Anthony Simion,‡ Becca Fleischer,* and Sidney Fleischer

ABSTRACT: We have quantitated bile acids and their conjugates in rat liver using high-pressure liquid chromatography. Over 95% of the hepatic bile acid pool in rat liver homogenates is present as taurocholate and tauromurichолate. Although over 60% of the bile acid pool is recovered in the supernatant, evidence is presented suggesting that taurocholate redistributes among the subcellular fractions during their isolation. Taurocholate (TC) binding to purified subcellular fractions from rat liver was determined by using equilibrium dialysis in a TC concentration range from 0.1 to 100 μM. This is well below the critical micellar concentration of taurocholate (3 mM). All of the fractions investigated exhibited low-affinity binding with dissociation constants from 80 to 240 μM as did membrane lipid vesicles. Therefore, low-affinity binding appears referable to taurocholate nonspecifically partitioning into the lipid bilayer. High-affinity binding is present in plasma membranes, Golgi, and cell supernatant. The high-affinity binding sites in Golgi have a mean dissociation constant (A_1) of 1.0 μM and bind 0.15 nmol of TC/mg of protein. Similarly, the high-affinity binding sites of plasma membrane have an A_1 of 1.3 μM and bind 0.15 nmol of TC/mg of protein. For cell supernatant, the A_1 was 4.8 μM, and 0.35 nmol of TC was bound per mg of protein. Mitochondria, smooth and rough microsomes, and Golgi liposomes showed no detectable amounts of high-affinity binding. These results are compatible with a role for the Golgi complex, cytoplasmic component(s), and plasma membranes in transhepatic bile acid transport.

Liver cells take up bile acids and conjugated bile salts from the intestines via the hepatic portal blood and secrete them into the bile as part of the enterohepatic circulation. Little is known concerning the intracellular pathway by which bile acids and bile salts are transported across the hepatocytes from the blood stream to the bile canaliculi. The transport process is rapid, since glycocholate injected into the superior meristemic vein appears in the bile after 1 min and reaches a maximum 2.5 min after injection (Strange et al., 1979a). This contrasts with a transit time of 15 min for the synthesis and secretion of serum albumin by hepatocytes (Peters et al., 1971).

The rapid transport of glycocholate through rat liver cells suggests that there is a specific intracellular transport mechanism for bile acids and conjugated bile salts. The proteins involved in a specific transport mechanism might be expected to bind bile acids with a higher affinity than observed for the nonspecific partitioning of bile acids into the lipid bilayers of membranous subcellular fractions. The enzymes that catalyze bile acid conjugation in the liver (bile acid:CoA ligase and bile acyl CoA:amino acid transferase) have Michaelis constants of less than 20 μM for bile acids and their derivatives (Simion et al., 1983a; Killenberg & Jordan, 1978; Vessey, 1979). This contrasts with the nonspecific binding of taurocholate to albumin, with a dissociation constant (K_d) of approximately 550 μM (Roda et al., 1982).

Intestinal flora cleave the peptide bond that conjugates the bile acid to either glycine or taurine and remove hydroxyl groups from the sterol ring of the bile acids, thereby decreasing the critical micellar concentration at physiological salt concentrations and pH (Helenius et al., 1979). The liver contains enzyme systems which rehydroxylate and reconjugate bile acids, thereby obviating possible damage by detergent action. Differences observed in the relative proportion of di- and trihydroxylated bile acids and their taurine conjugates in isolated subcellular fractions might help position the organelle in the intracellular transport pathway analogously to previous studies of the subcellular distribution of nascent and mature forms of albumin or α-fetoprotein (Edwards et al., 1976; Belanger et al., 1979) which helped confirm the intracellular pathway for synthesis and secretion of serum proteins by hepatocytes.

In the present study, the distribution of bile acids and conjugated bile salts in highly purified subcellular fractions was determined. This was achieved by developing analytical methods utilizing high-pressure liquid chromatography for quantitation of bile acids and their glycine and taurine conjugates. The subcellular distribution of taurocholate binding sites was determined by using equilibrium dialysis with the view that subcellular fractions involved in intrahepatic bile acid transport might be expected to contain high-affinity binding sites for bile acids.

Materials and Methods

Specialized reagents were obtained as follows. Radioactive [^{3}H]- and [^{14}C]taurocholic acid, [^{14}C]cholic acid, and [^{3}H]-deoxycholic acid were obtained from New England Nuclear (Boston, MA); nonradioactive bile acids and bile salts and tris(hydroxymethyl)aminomethane (Tris)[1] (Trizma base) were from Sigma Chemical Co. (St. Louis, MO); 3α-hydroxysteroid dehydrogenase was from Worthington Biochemicals (Freehold, NJ); special enzyme grade sucrose was from E. M. Labs (Elmsford, NY); methanol and acetonitrile (UV grade) were from Burdock and Jackson (Muskegan, MI). All other materials used were of reagent grade quality, and aqueous solutions were made with deionized water. The elution buffers for high-pressure liquid chromatography (HPLC) were made with glass-distilled, deionized water to prevent any hydrophobic

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‡ Present address: Colgate-Palmolive Research Center, Piscataway, NJ.

[1] Abbreviations: TC, taurocholate; HPLC, high-pressure liquid chromatography; DOCA, deoxycholic acid; Tris, tris(hydroxymethyl)-aminomethane; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid.

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material leached from the ion-exchange column from absorbing to the reverse-phase HPLC column (Brewer, 1974).

Preparation of Subcellular Fractions. Cell supernatant, "total" plasma membranes, total microsomes, rough and smooth microsomes, and mitochondria were prepared from the same pooled rat liver homogenates by the method of Fleischer & Kervina (1974). The "total" plasma membrane fraction contains membranes derived from all three domains of the cell surface—sinusoidal, contiguous (cell–cell junctions), and bile canaliculus (Hubbard et al., 1983). Bile canalicular plasma membrane was prepared as described by Kramer et al. (1982), although to retain the distinctive morphology of the bile canaliculus, only the initial sucrose gradient was used. Golgi complex was isolated directly from the total homogenates by using either H_2O–sucrose or D_2O–sucrose step gradients as described by Fleischer (1981). The purity of the subcellular fractions was estimated with the use of marker enzymes (Fleischer & Kervina, 1974; Simion et al., 1983a). The protein concentrations of the subcellular fractions were determined by the method of Lowry et al. (1951), using bovine plasma albumin as a standard.

Quantitation of Bile Acids and Conjugated Bile Salts in Subcellular Fractions Using High-Pressure Liquid Chromatography. The bile acid and conjugated bile salt contents of total homogenate and purified subcellular fractions from rat liver were determined by HPLC. The bile acids and conjugated bile salts were extracted from the samples with 95% ethanol containing 0.1% ammonia and purified by the method of Sandberg et al. (1965), except that Dowex AG 1X2 resin was used instead of Amberlite XA2 (Kuron & Tennent, 1961). Impurities in the Dowex, which coelute from the anion-exchange column with the bile acids and conjugated bile salts, had been previously removed by repeatedly washing the resin in bulk with glass-distilled methanol followed by acetonitrile (HPLC grade). After the Dowex column was washed with 2 column volumes (20 mL) of 95% ethanol followed by 2 volumes of 1:1 95% ethanol:1,2 dichloroethane and then 2 volumes of 80% ethanol, the bile acids and conjugated bile salts were eluted from the Dowex by 12 volumes (120 mL) of 0.2 M ammonium carbonate in 80% ethanol. The eluate was evaporated to dryness under reduced pressure and then resuspended in a minimal volume of elution buffer (22% acetonitrile in 4 mM phosphoric acid, pH 2.5). Aliquots of the sample were then chromatographed by using a Perkin-Elmer series 3 HPLC equipped with a reverse-phase ODS-HC SIL-X-1 column, 4.6 mm × 25 cm. The conjugated bile salts were eluted from the column by 22% acetonitrile in 4 mM phosphoric acid and were detected by their absorbance at 198 nm with a Perkin-Elmer LC55 spectrophotometer equipped with an 8-μL flow cell or at 195 nm with an LDC spectromonitor III (Riveria Beach, FL) equipped with a 15-μL flow cell. The conjugated bile salts were eluted from the column with retention volumes similar to those reported by Shaw et al. (1978). The bile salt peaks were quantitated by comparing areas to standard curves produced by chromatographing known quantitites of bile salt standards under similar conditions.

Unconjugated bile acids were eluted by 40% acetonitrile in 3 mM phosphoric acid, and fractions were collected. With this elution solvent, unconjugated bile acids do not have sufficient UV absorption to be detected directly during elution. This is in contrast to elution with 75% MeOH as reported by Armstrong & Carey (1982). In order to identify and quantitate the bile acids in our elution, we used the 3-hydroxysteroid dehydrogenase assay described by Turley & Dietschy (1978) with the reaction volume reduced to 1 mL. The assay was also used to confirm that the peaks identified by their retention times were indeed bile salts.

Equilibrium Dialysis. Equilibrium dialysis was used to characterize the binding of taurocholate to the purified subcellular fractions. Dialysis chambers with a volume of 250 μL were used in this study. The chambers were divided into two parts by a dialysis membrane with a cutoff of 3500 daltons. The dialysis membrane was washed as described by Brewer (1974). The subcellular fraction in 0.1 M Tris-HCl and 0.25 M sucrose, pH 8.0 (at 4 °C), was introduced into one side of the dialysis chamber, while an equal volume (100 μL) of buffer without protein was added to the other side of the chamber. Then an equal amount of [^{14}C]taurocholate (3–100 dpm/pmol) was added to either side of the chamber. The concentration of taurocholate used varied from 0.1 to 100 μM. The dialysis chambers were then loaded onto a drum which rotated at 6 rpm. Dialysis required 24 h to achieve equilibrium at 4 °C. At that time, the distribution of radioactivity and protein on either side of the dialysis membrane was determined. The radioactivity in 75-μL aliquots drawn from either side of the dialysis membrane was determined after mixing with 10 mL of ACS scintillation fluid and counting in a Mark III liquid scintillation counter. The protein concentration was determined by the method of Lowry et al. (1951).

Measurement of the Rates of Binding and Dissociation of Taurocholate by Purified Subcellular Fractions. The binding rate of taurocholate to rough microsomes was measured as follows: rough microsomes were preincubated in the buffer used to homogenize rat liver and prepare microsomes [0.25 M sucrose containing 10 mM Hepes, pH 7.5 (Fleischer & Kervina, 1974)] for 15 min at 4 °C, at a protein concentration of 0.2 mg/mL. A small aliquot of [^{3}H]taurocholate (in 0.25 M sucrose) was added to give a final taurocholate concentration of 80 μM, and the protein suspension was vigorously mixed. At given times, aliquots of the protein suspension were removed and rapidly filtered using 0.22-μm Millipore filters which had been prewashed with 5 mL of Hepes/sucrose homogenization buffer. Filtration was carried out by using an Amicon vacuum manifold connected to a vacuum of 680 mmHg. The microsomes were filtered dry practically instantaneously. The filters were then rewashed with 5 mL of homogenization buffer, dried by blotting on tissue paper, then dissolved in 2 mL of ethylene glycol methyl ether (scintillation grade), and counted after addition of 10 mL of ACS scintillation fluid.

The rate of dissociation of taurocholate from rough microsomes was determined as follows: rough microsomes were preincubated at 4 °C for 1 h in homogenization buffer (0.25 M sucrose and 10 mM Hepes, pH 7.5) containing 80 μM [^{3}H]taurocholate at 3.8 mg of protein/mL. They were then diluted into 19 volumes of taurocholate-free homogenization buffer, which was rapidly stirred at 4 °C, to give a protein concentration of 0.2 mg/mL. At given times, aliquots of the diluted rough microsomes were rapidly filtered with 0.22-μm Millipore filters that had been prewashed with 5 mL of homogenization buffer. The filters were rewashed with 5 mL of homogenization buffer, and the taurocholate bound to the rough microsomes was quantitated as before.

The rate of dissociation of bound taurocholate from Golgi complex was determined in a similar manner to that of the rough microsomes. Golgi complex was incubated overnight at 4 °C at a concentration 2.6 mg of protein/mL of 0.1 M Tris-HCl, pH 8.0, containing 0.25 M sucrose and 40 μM [^{14}C]taurocholate. An aliquot of the Golgi mixture was then diluted into 29 volumes of taurocholate-free buffer of the same

Table I: Retention Times of Unconjugated Bile Acids Determined by Reverse-Phase HPLC[a]

unconjugated bile acid	retention time (min)	unconjugated bile acid	retention time (min)
β-muricholate	6.8 ± 0.2	chenodeoxycholate	35.6 ± 1.7
cholate	11.4 ± 0.5	deoxycholate	37.3 ± 0.5
hyodeoxycholate	15.3 ± 0.3	lithocholate	132 ± 8

[a] Retention times are given as a mean of three values (±standard deviation). The retention time for the unconjugated bile acids was determined by reverse-phase HPLC as described under Materials and Methods. The bile acids were eluted from an ODS-HC/SIL-X-1 column by using 40% acetonitrile in aqueous 3 mM phosphoric acid, with a flow rate of 0.2 mL/min.

composition which was rapidly stirred at 4°C. At given times, aliquots of the diluted Golgi were rapidly filtered by using 0.45-μm Millipore filters which has been prewashed with 5 mL of the Tris/sucrose buffer. The filters were then rewashed with 5 mL of Tris/sucrose buffer and dried, and the radioactivity bound to the Golgi complex was determined as described above.

Results

Bile acids and their taurine- and glycine-conjugated derivatives can be identified and quantitated independently from each other by using reverse-phase HPLC. The elution scheme is based on that of Shaw et al. (1978). Although 22% acetonitrile was used to elute the conjugated bile salts instead of 34% propan-2-ol, the relative retention times of the conjugated bile salts are similar to those reported by these authors.

We have extended the use of HPLC to separate the unconjugated bile acids. These are eluted from the reverse-phase column by 40% acetonitrile in 3 mM phosphoric acid. The relative retention times are shown in Table I. Unlike the conjugated bile salts, the unconjugated bile acids do not absorb ultraviolet light at 180–200 nm. Instead, they were quantitated by using the 3α-hydroxysteroid dehydrogenase assay.

Analysis of the bile acids present in the total homogenate by HPLC shows that more than 95% of the bile acid pool in rat liver is present as the taurine conjugates taurocholate and tauromuricholate (Table II). The recovery of taurocholate was determined either by spiking the sample with a small amount of [^{14}C]taurocholate (1–10 nmol, 100 dpm/pmol) or by adding larger amounts of nonradioactive taurocholate (500 nmol). A mean recovery of 87 ± 4% (n = 4) was achieved, even at the lower limit. There were essentially negligible amounts of unconjugated bile acids (such as cholate, chenodeoxycholate, or DOCA) or glycine conjugates of the bile acids found in the rat livers. The limits of detectability were 3 μg of cholate or 1 μg of DOCA per g of liver. The absence of unconjugated bile acids in homogenates is not due to a failure to recover the bile acids from the samples, as the recovery of cholate was shown to be 88% and that of DOCA 91%. A control was run to test whether conjugation of radioactive cholate or DOCA could occur in the homogenate. No conjugation was detected when these bile acids were added to the

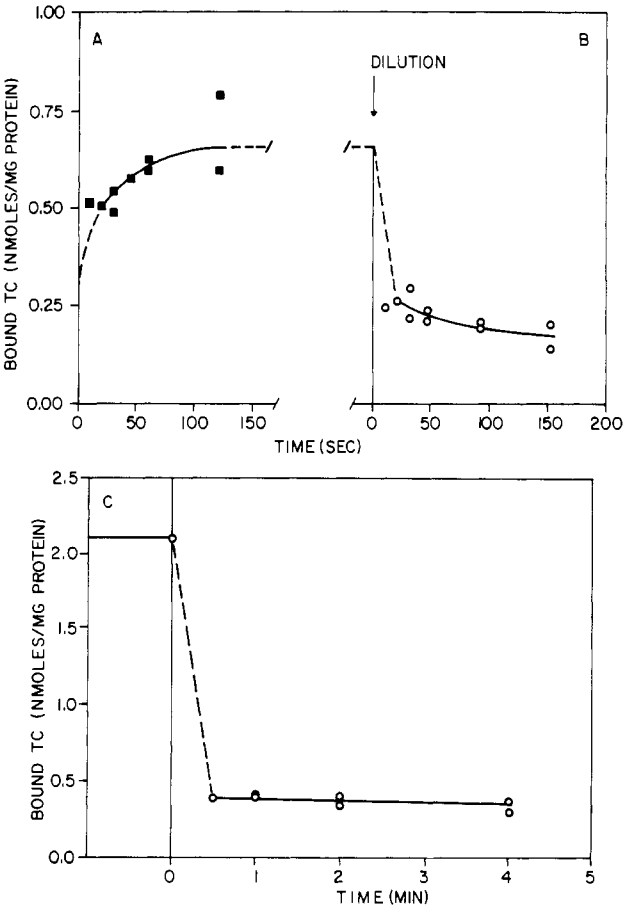
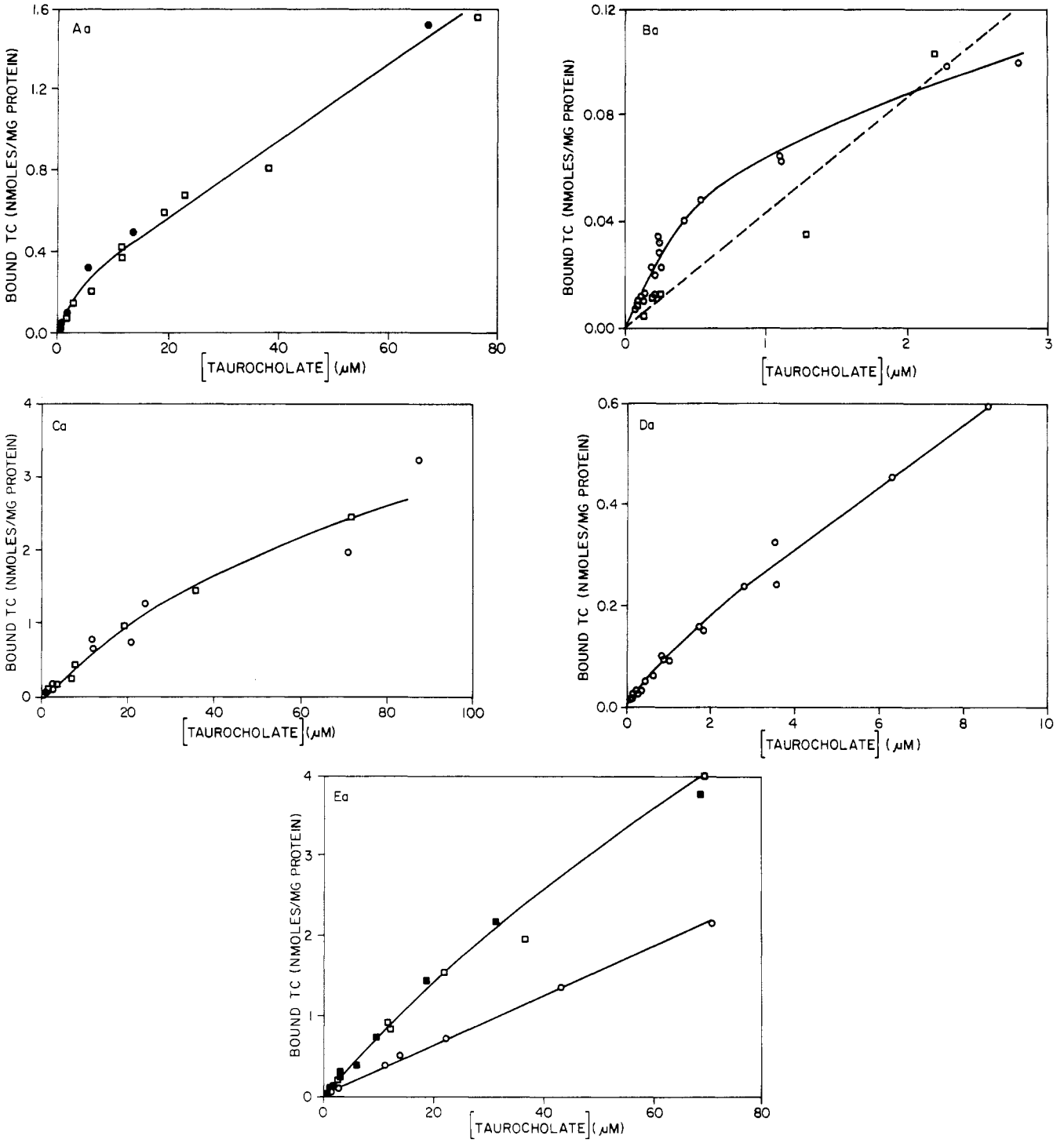


FIGURE 1: Rapid binding and dissociation of taurocholate to some purified subcellular fractions. (A) Measurement of the rate of binding of taurocholate to rough microsomes. The rough microsomes were incubated in 0.25 M sucrose containing 10 mM Hepes, pH 7.5 at 4 °C, at a protein concentration of 0.2 mg/mL. A small aliquot of [^{3}H]taurocholate was added to a final concentration of 80 μM. After addition of radioactivity, aliquots of the protein suspension were filtered, and the taurocholate bound to rough microsomal protein was determined as described under Materials and Methods (■). (B) The rate of dissociation of taurocholate from rough microsomes was determined by diluting rough microsomes that had been preincubated in 80 μM taurocholate into 19 volumes (○) of taurocholate-free buffer to give final protein concentrations of 0.2 mg/mL. At set times, aliquots of the protein suspension were rapidly filtered, and the taurocholate bound to the microsomes was determined. (C) Dissociation rate of taurocholate binding from Golgi complex. Golgi complex was equilibrated with 40 μM taurocholate at 4 °C as described under Materials and Methods. At time zero, the vesicles were diluted 29-fold in isoosmotic buffer, filtered, and washed as described. In less than 30 s after dilution, a much lower new equilibrium value of bound taurocholate was reached. Similar results are observed with 0.1 M sodium phosphate, pH 7.1, which is the phosphate concentration used in the homogenization of rat liver to prepare the Golgi fraction.

Table II: Distribution of Bile Salts in Rat Liver[a]

	taurocholate			tauromuricholate		
fraction	μg/g of liver	μg/mg of protein	% of homogenate pool	μg/g of liver	μg/mg of protein	% of homogenate pool
homogenate	35.8 ± 14.1	0.176 (4)[b]	100.0	20.0 ± 5.7	0.157 (3)	100.0
cell supernatant	21.9 ± 1.9	0.369 (2)	61.2	17.2 ± 1.4	0.285 (2)	86.0
total microsomes	3.1 ± 1.6	0.184 (3)	8.6	0.8 ± 0.3	0.060 (2)	4.0
rough microsomes	<0.1	0.030 (2)	[c]	ND[d]		
D_2O Golgi	<0.2	0.060 (4)	[c]	ND[d]		

[a] No taurodeoxycholate, taurochenodeoxycholate, glycine-conjugated bile salts, or unconjugated bile acids were detected (limit of detectability in homogenate, 3 μg/g of liver). [b] Numbers in parentheses indicate number of preparations analyzed. [c] Negligible. [d] ND, not determined.



homogenate prior to extraction and analysis.

The bile acid and conjugated bile salt contents of highly purified subcellular fractions are summarized in Table II. Cell supernatant and the total microsomal fraction were found to contain significant levels of taurocholate and tauromuricholate. As with homogenate, no unconjugated bile acids, glycine-conjugated bile salts, taurochenodeoxycholate, or taurodeoxycholate could be detected. We were unable to detect any bile acids or conjugated bile salts in the rough microsomes or Golgi complex using HPLC. This was confirmed by using the 3α-hydroxysteroid dehydrogenase assay directly on the ethanolic extract from either fraction.

The rate of binding and dissociation of taurocholate to rough microsomes was determined by filtration (Figure 1A,B). Both processes are very rapid and appear to be complete within 2 min at 4 °C. As binding and dissociation processes are essentially instantaneous compared to the 2 h required to separate the rough microsomes from the total microsomal fraction, any taurocholate which had been bound to the rough microsomes would have had ample opportunity to equilibrate with the taurocholate-free media and so would be removed from the microsomes. Similarly, the rate at which the binding of taurocholate to Golgi reaches a new equilibrium after dilution is faster than the filtration procedure (15 s, Figure 1C). Since Golgi complex is prepared by a 65-min centrifugation through a sucrose gradient devoid of taurocholate, any taurocholate originally bound to the Golgi would also be washed away.

When radioactive taurocholate is added to the homogenate prior to fractionation, the observed distribution of exogenous radioactive label is similar to that of the endogenous taurocholate pool (Table III). This suggests that the observed distribution of endogenous taurocholate could be due to the reequilibration of the taurocholate pool during subcellular fractionation.

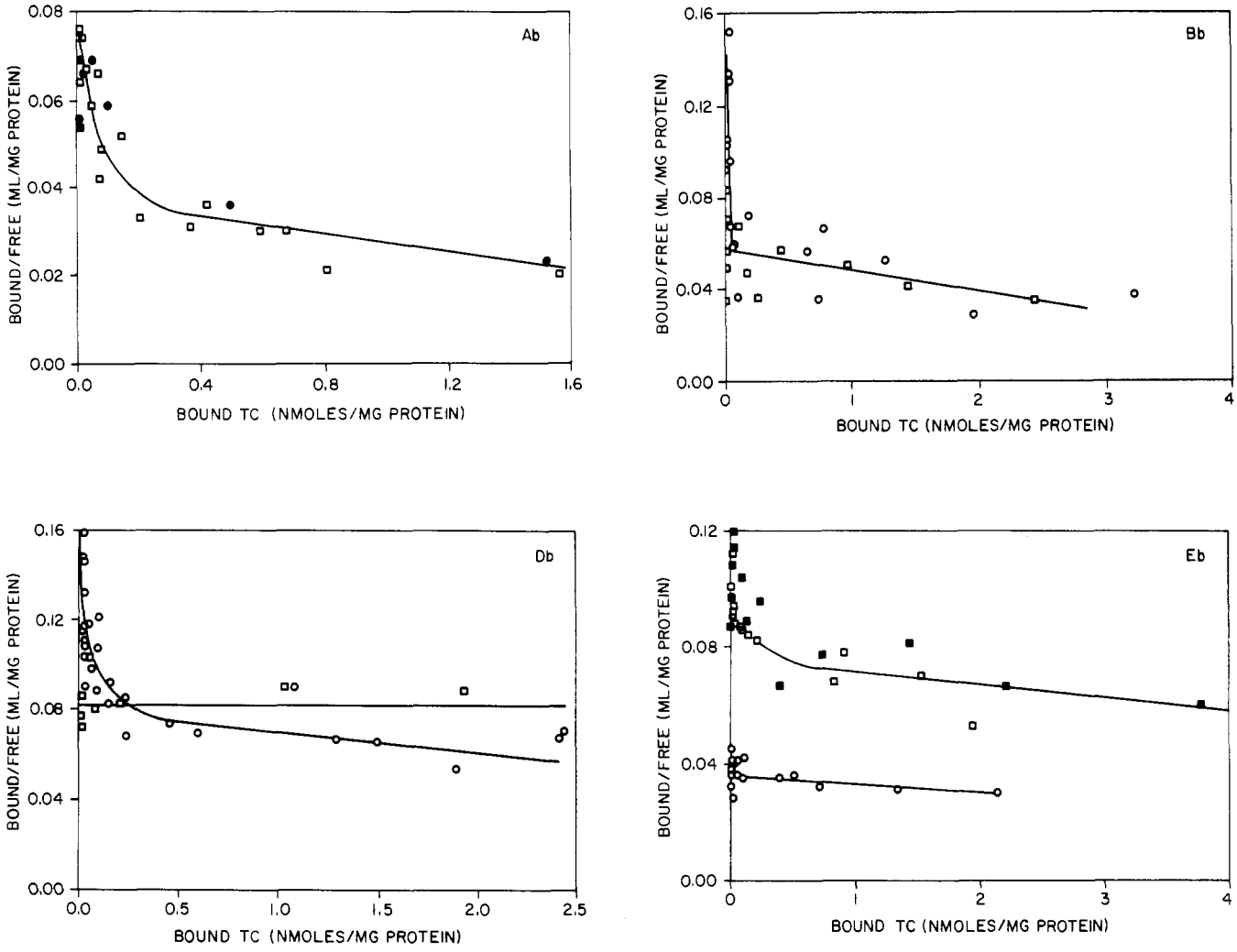


FIGURE 2: Binding of taurocholate by purified subcellular fractions of rat liver. Binding was determined by equilibrium dialysis for 24 h at 4 °C in 0.1 M Tris-HCl and 0.25 M sucrose, pH 8.0, as described under Materials and Methods. Binding data for the subcellular fractions investigated are presented in two ways: (a) The nanomoles of taurocholate bound per milligram protein or microgram lipid phosphorus are plotted against the free taurocholate concentration. (b) The data obtained are plotted according to Scatchard. (A) Cell supernatant (□, two preparations). In addition, a sample of cell supernatant was dialyzed twice against 30 volumes of buffer for 24 h to remove endogenous taurocholate before binding measurements were carried out (●). (B) Plasma membranes. Two types of preparations were analyzed. Total plasma membranes, which contain portions of each of the three domains of the hepatocyte cell membrane, were prepared by the method of Fleischer & Kervina (1974) (○, two preparations). A second preparation, consisting predominantly of bile canalicular plasma membranes (Kramer et al., 1982), is also shown (□, one preparation). (C) Plasma membranes prepared as described in (B). Higher concentrations of taurocholate are shown. In this range, there is no significant difference in the binding curves for the two preparations. (D) Golgi apparatus (○, four preparations). The Scatchard plot (Figure 2Db) also shows TC binding to liposomes prepared from the lipid extract of a Golgi preparation (□). Before extraction, the Golgi complex contained 28.7 μg of phosphorus per mg of protein, so that binding of TC to liposomes is expressed on the basis of 28.7 μg of phosphorus = 1 mg of protein. (E) Mitochondria (○, two preparations), rough microsomes (□, two preparations), and smooth microsomes (■, one preparation).

The binding of taurocholate to purified subcellular fractions of rat liver was determined by equilibrium dialysis. All the subcellular fractions studied (rough and smooth microsomes, cell supernatant, mitochondria, Golgi complex, and plasma membranes) were capable of binding taurocholate. Plots of taurocholate bound per milligram of protein against the free taurocholate concentration for each of the subcellular fractions studied are shown in Figure 2a. Golgi complex, cell supernatant, and total plasma membranes each appear to contain a component which saturates at low substrate concentrations (below 10 μM). Rough and smooth microsomes, mitochondria, and bile canalicular plasma membranes lack a significant amount of this component as taurocholate binding shows a linear relationship to the free bile acid concentration at low concentrations.

The binding data were replotted according to the method of Scatchard (Figure 2b). The Scatchard plots for the Golgi apparatus, cell supernatant, and the total plasma membrane fraction clearly show more than one component. It is possible to fit these data to a model for two binding classes. The Golgi complex and total plasma membranes each have "high"-affinity binding sites which bind 0.15 nmol of taurocholate per mg of protein with mean dissociation constants (A_1) of about 1 μM. The cell supernatant binds 0.4 nmol of TC per mg of protein with a mean dissociation constant of 5 μM (Table IV). Furthermore, each subcellular fraction has a larger group of "low"-affinity binding sites which have mean dissociation constants (A_2) ranging from 80 μM for cell supernatant to 100 μM for Golgi complex.

Liposomes made from Golgi membranes by the method of Fleischer & Fleischer (1967) show only the "low-affinity" class of binding sites, suggesting that "low-affinity" binding represents the interaction of taurocholate with membrane lipids. Although there appears to be the suggestion of a small amount

Table III: Correlation between the Distribution of Endogenous and Added Taurocholate[a]

subcellular fraction	endogenous taurocholate (determined by HPLC)	no. of prepn	exogenous taurocholate (added [^{14}C]TC)	no. of prepn
cell supernatant	61.2 ± 5.3	2	57.2 ± 5.7	2
total microsomes	8.6 ± 4.4	3	9.0 ± 2.7	2
rough microsomes	<0.3	2	0.4	1
Golgi	<0.6	3	0.5 ± 0.2	2

[a] The distribution is expressed as (taurocholate in fraction)/(taurocholate in homogenate) × 100. Cell supernatant and total microsomes were prepared from the same postmitochondrial supernatant by centrifugation at 100000*g* for 1 h (Fleischer & Kervina, 1974). Rough microsomes were prepared from the total microsomal fraction as previously described and contain approximately 70% of the total microsomal protein. Golgi complex was prepared by the method of Fleischer (1981). Endogenously added taurocholate in the subcellular fractions was quantitated by HPLC Materials and Methods and Table II). Ten nanomoles (10^6 dpm) of [^{14}C]taurocholate (the exogenous label) was added to the homogenate prior to fractionation. The radioactivity in each subcellular fraction was determined by scintillation counting, after the fraction had been incubated in Protosol (New England Nuclear, Boston, MA) for 3 h at 37 °C.

Table IV: Taurocholate Binding to Subcellular Fractions of Rat Liver[a]

fraction	N_1 (nmol/mg of protein)	A_1 (μM)	N_2 (nmol/mg of protein)	A_2 (μM)	no. of prepn
cell supernatant	0.35 ± 0.06	4.8 ± 0.9	3.0 ± 0.3	79 ± 7	2
rough microsomes	<0.02[b]		8.9 ± 1.6	102 ± 19	2
smooth microsomes	<0.02[b]		9.2 ± 1.2	94 ± 16	2
Golgi complex	0.15 ± 0.03	1.0 ± 0.3	8.9 ± 1.9	99 ± 21	4
mitochondria	<0.02[b]		9.0 ± 1.9	238 ± 51	2
plasma membranes					
total	0.15 ± 0.02	1.3 ± 0.2	5.4 ± 1.6	91 ± 28	2
bile canalicular	<0.02[b]		6.8 ± 3.2	116 ± 55	1

[a] Binding of taurocholate to the subcellular fractions was determined by equilibrium dialysis in 0.1 M Tris-HCl and 0.25 M sucrose, pH 8.0 at 4 °C, as described under Materials and Methods. When the amount of taurocholate bound per milligram protein is plotted against the free taurocholate concentration (Figure 2), cell supernatant, total plasma membrane, and Golgi complex show a saturable binding component discernible at low taurocholate concentrations. To estimate the binding constants, the direct binding data were fitted to a model which assumed that there were two groups of binding sites using the algorithm of Bevington (1969). That is $[TC]_{bound} = N_1[TC]_{free}/(A_1 + [TC]_{free}) + N_2[TC]_{free}/(A_2 + [TC]_{free})$ where A_1 and A_2 are mean dissociation constants for the two groups of binding sites and N_1 and N_2 represent the total binding capacity of each group of binding sites. [b] The data for rough and smooth microsomes, mitochondria, and bile canalicular plasma membranes were assumed to possess only one class of binding sites. The binding constants were estimated by fitting the Scatchard plot data to a straight line.

of tight binding in the Scatchard plots for rough and smooth microsomes, these data as well as those for mitochondria and bile canalicular plasma membranes are best fitted to straight lines representing a single class of low-affinity binding with mean dissociation constants (A_2) ranging from 90 μM for smooth microsomes to 240 μM for mitochondria and binding capacities (N_2) of 7–9 nmol of TC/mg of protein. All measured dissociation constants are significantly lower than the critical micellar concentration of taurocholate, which is approximately 3 mM (Helenius et al., 1979).

The weak binding of taurocholate to the subcellular fractions can also be described as partitioning of the taurocholate between the lipid phase of the membrane and the aqueous medium. For ideal mixing, the distribution can be expressed as

$$X_{TC,H_2O} = X_{TC,lipid}(cmc)^\circ_{TC}$$

where X_{TC,H_2O} is the mole fraction of taurocholate free in solution, $X_{TC,lipid}$ is the mole fraction of taurocholate bound to the lipid phase of the membranes, and $(cmc)^\circ_{TC}$ is the critical micellar concentration of taurocholate (Tanford, 1980). When our low-affinity binding data are expressed in this manner, all the subcellular fractions gave linear relationships. The $(CMC)^\circ_{TC}$ values obtained, by using the total lipid contents of the subcellular fractions (Zambrano et al., 1975), were 8.0×10^{-5} for mitochondria, 8.8×10^{-5} for rough endoplasmic reticulum, 7.9×10^{-5} for Golgi, 10.6×10^{-5} for Golgi liposomes, and 12.4×10^{-5} for plasma membranes. These values are very similar to each other and to the reported cmc for taurocholate of 5.4×10^{-5} (Helenius, 1979). This supports the view that the weak binding represent partitioning of the taurocholate between the buffer and the lipids of the subcellular fractions.

The concentration of endogenous taurocholate in each of the subcellular fractions studied might be expected to affect the observed binding curves obtained by diluting the added radioactive taurocholate. Only the cell supernatant contains a measurable concentration of bile acids (Table II). To remove the endogenous taurocholate, cell supernatant was dialyzed for 24 h against 60 volumes of dialysis buffer. The binding characteristics of the dialyzed cell supernatant appeared to be similar to those of cell supernatant which had not been dialyzed. Strange et al. (1976) made a similar observation when measuring the binding of cholic acid to cell supernatant. No bile acids or conjugated bile salts could be detected in rough microsomes or Golgi complex. Since the preparation of plasma membrane, smooth microsomes, and mitochondria involves multiple washes in sucrose solutions which lack taurocholate, any endogenous taurocholate would have been leached away from the membrane fraction. Therefore, all the membranous subcellular fractions studied contain essentially no endogenous bile acids to affect the observed binding parameters.

Discussion

This study describes the distribution of bile acids and conjugated bile salts in rat liver. Reverse-phase HPLC was used to quantitate the bile acids and their taurine and glycine conjugates. We found that over 95% of the bile acid pool in rat liver is in the form of the conjugated trihydroxylated bile salts taurocholate and tauromuricholate. This is the first direct demonstration that the vast majority of the intracellular hepatic pool is in the form of conjugated bile salts, previous studies being limited by gas–liquid chromatography (GLC) methodology. This observation is consistent with our recent findings that the enzyme which catalyzes the rate-determining step of conjugation, bile acid:CoA ligase, is 3-fold more active than previously reported (Simion et al., 1983a). Furthermore, Bjorkhem et al. (1974) showed that, in vitro, conjugated dihydroxylated bile salts are hydroxylated more rapidly than the corresponding unconjugated bile acid. Conjugation of bile acids has an important role in hepatic physiology. The critical micellar concentration of conjugated bile salts is higher than that of the unconjugated bile acids at physiological pH and salt concentration (Helenius et al., 1979), so the conjugated

bile salts are less likely to act as detergents which would damage the hepatocytes.

The methodology developed to use reverse-phase HPLC to separately quantitate individual bile acids and conjugated bile salts from a complex tissue such as rat liver represents a significant advance over using gas–liquid chromatography, where the conjugated bile salts must be deconjugated prior to analysis, so that the relative amounts of a bile acid and its conjugates cannot be separately determined. Previously, Shaw & Elliot (1978) used reverse-phase HPLC to show that bile from rats contained a low level of taurochenodeoxycholate as well as taurocholate and tauromuricholate. However, the presence of unconjugated bile acids in rat bile was not investigated. Essentially all bile acids in human bile are in the conjugated form. No unconjugated bile acids were detected after derivatization with *p*-bromophenacyl bromide (Mingrone & Greco, 1980).

We have studied the distribution of the bile acid pool between well-defined subcellular fractions of rat liver. Most is recovered with the cell supernatant (taurocholate 61%, tauromuricholate 86%; Table II), although a small proportion (4–9%) is recovered from the total microsomal fraction. The conjugated bile salts are lost from rough microsomes during their isolation from the total microsomal fraction. Golgi complex, which is prepared from liver homogenate by flotation through a sucrose gradient, is also essentially devoid of bile acids and bile salts. Direct measurements under conditions used for subcellular fractionation show that binding and dissociation of taurocholate to membranous organelles are faster than 15 s, which is negligible compared to the time required to prepare subcellular fractions. Hence, endogenous taurocholate originally associated with the membranous fractions would be leached from the organelles during their isolation, suggesting that after homogenization the bile acid pool reequilibrates among the subcellular fractions of the liver, according to the relative ability of each fraction to bind or partition the taurocholate. This conclusion is supported by our observation that exogenous radiolabeled taurocholate is distributed between the subcellular fractions in similar proportions to endogenous taurocholate (Table III).

Previously Okishio & Nair (1966) reported that 70% of the bile acid pool was recovered in the cell supernatant with the rest of the pool distributed among the membranous subfractions. Strange et al. (1977) made a similar observation, although they showed indirect evidence that the observed subcellular distribution might not reflect that in vivo (Strange et al., 1979).

The rapid reequilibration of the bile acid pool after homogenization of the liver tissue prevents pulse–chase techniques from being used to study the intracellular pathway for bile salt transport. An indirect aproach to elucidating the involvement of intracellular organelles in bile acid metabolism and transport is to study the distribution of the metabolic and transport processes in purified subcellular fractions (Simion et al., 1983a–c). Many of the bile acid metabolizing enzymes and transport proteins appear to be intrinsic membrane proteins so that they are not readily displaced from their original subcellular location.

This paper presents the first comprehensive survey of the distribution of taurocholate binding sites in purified subcellular fractions of rat liver under comparable conditions, which allows direct comparisons to be made between the different fractions. The fractions can be divided into two groups. The first, consisting of Golgi complex, cell supernatant, and total plasma membrane fractions, exhibits two classes of binding sites, including sites which are able to bind taurocholate tightly (A_1 = 5 μM). The second group, consisting of rough and smooth microsomes, mitochondria, and bile canalicular plasma membranes, does not have a significant number of tight binding sites and only shows the "weaker" binding (A_2 = 70 μM) exhibited by all the subcellular fractions. Tight binding can be discriminated from partitioning into the lipid bilayer since the weaker binding is manifest in lipid vesicles (Figure 2). However, we have no evidence that the high-affinity binding sites of Golgi complex, cell supernatant, and plasma membranes are due to the same proteins.

Accatino & Simon (1978) reported that plasma membrane has a cholic acid binding site with a dissociation constant of 1.4 mM, a value more comparable with nonspecific partitioning of bile acids into the lipid bilayer. Anwer et al. (1977) reported that there were high-affinity binding sites for cholic acid in plasma membranes, with a dissociation constant (K_d) which increases with decreasing temperature to 0.3 μM at 10 °C.

The high-affinity binding sites in plasma membranes may have a role in sodium-stimulated uptake of taurocholate by hepatocytes (Schwarz et al., 1975; Anwer & Hegner, 1978). Total plasma membranes, which are capable of sodium gradient stimulated taurocholate uptake, also exhibit high-affinity binding while bile canalicular plasma membranes, which do not transport taurocholate, also do not have a detectable level of high-affinity binding sites (Table IV; Simion et al., 1983c).

There have been several reports characterizing the binding of bile acids to cell supernatant proteins (Strange et al., 1976, 1977) or proteins derived from that subcellular fraction (Sugiyama et al., 1983). Strange et al. (1976) showed that cholic acid binding to cell supernatant possessed a class of binding sites which had a dissociation constant of 14 μM, as well as a much larger number of low-affinity binding sites. Purified cytoplasmic proteins from rat liver such as glutathione *S*-transferases A and B and bile acid binders I and II have dissociation constants for taurocholate that range from 120 μM to 3 mM, suggesting that these purified proteins are not those responsible for the high-affinity taurocholate binding observed in unfractionated cell supernatant.

The Golgi complex has been implicated in taurocholate transport on the basis of morphological evidence. Jones et al. (1979) observed that when amounts of taurocholate transported through rat liver cells increased, Golgi complex was observed to proliferate around the bile canaliculi.

Some subcellular fractions have too few high-affinity binding sites to be detected by equilibrium dialysis under our experimental conditions. Smooth microsomes are capable of taking up taurocholate (Simion et al., 1983c), while cholic acid:CoA ligase is localized in the rough and smooth microsomes (Simion et al., 1983a). Equilibrium dialysis, however, does not detect a significant number (<0.02 nmol of TC bound/mg of protein) of high-affinity binding sites in either fraction.

Registry No. Taurocholic acid, 81-24-3; tauromuricholic acid, 52021-72-4.

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Exciton Interaction in Allophycocyanin†

Karoly Csatorday,* Robert MacColl, Vilmos Csizmadia, Jozef Grabowski,‡ and Csaba Bagyinka

ABSTRACT: The absorption and circular dichroism (CD) spectra of allophycocyanin II in the trimer and monomer (dissociated) forms were resolved into four and two components, respectively. The short-wavelength region of the visible spectra was approximated by a chimera of Lorentzian- and Gaussian-shaped bands having a bandwidth of ca. 65 nm. The rest of the bands have a pure Gaussian form. The characteristic 652-nm band in the absorption spectrum (656 nm in the CD spectrum) is shown to arise from exciton interaction between two fluorescent phycocyanobilin chromophores.

Allophycocyanin (APC) is that biliprotein that occupies the core of the light-harvesting phycobilisome and is in close association with the thylakoid membrane. It has been shown to exist in four spectral forms, APC I, II, III, and B (Gantt, 1981). From spectroscopic studies it has been inferred that APC II and III transmit excitation energy to the terminal emitters APC I and B (Gantt, 1980). The spectral characteristics of APC were discussed by MacColl et al. (1980), and the conclusion was drawn that the 652-nm absorption band of the trimer was due to intermediate-strength interaction between its chromophores. Allophycocyanin is composed of two subunits, α and β, each containing a single phycocyanobilin chromophore (Brown et al., 1975).

The absorption spectra of the individual APC trimers have been resolved into several Gaussian curves by computer analysis (Zilinskas et al., 1980). The absorption spectra of allophycocyanin "trimer majority" and "monomer majority" have also been similarly analyzed (Mimuro et al., 1982). In

† From the New York Department of Health, Center for Laboratories and Research, Albany, New York 12201 (K.Cs. and R.M.), and the Biological Research Center, Szeged, Hungary H-6701 (V.C., J.G., and C.B.). *Received November 1, 1983; revised manuscript received June 13, 1984.* This research was supported by the National Institute of General Medical Science, PHS/HHS GM 26050.

* Address correspondence to this author at Cogswell Laboratory, Rensselaer Polytechnic Institute, Troy, NY 12181.
‡ Permanent address: Institute of Environmental Engineering of Poznan Technical University, Poland.

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