papers and notes on methodology

Rapid isolation of vesicular and micellar carriers of biliary lipids by ultracentrifugation

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Abstract A simple, rapid, and new method has been developed to isolate and to quantitate the vesicular carrier of biliary lipids by isopycnic ultracentrifugation. The method combines the use of Metrizamide, as an inert centrifugation media to change the density of bile for isopycnic separation of vesicles, and a vertical rotor, to decrease both the time of centrifugation and the pressure of the hydrostatic column in the ultracentrifuge tube. Vesicles harvested from bile-Metrizamide density gradients were identified by negative staining electron microscopy. The buoyant densitites of biliary vesicles varied between 1.010 and 1.030 g/ml. The diameter of vesicles in fractions with $d < 1.020$ g/ml was 82 ± 10 nm and in fraction with $d \sim 1.030$ g/ml was 57 ± 8 nm. Gel filtration chromatography with Ultrogel AcA 34 was used to validate the quantitive isolation of vesicles by the ultracentrifugal method. In experiments with bile-Metrizamide continuous preformed density gradients, >93% of vesicular cholesterol was found in fractions with $d < 1.030$ g/ml after 285 min of centrifugation at 50,000 rpm in a VTi vertical rotor (Beckman Instruments, Inc.). When 16% Metrizamide was dissolved in bile and centrifuged for 120 min, >96% of total vesicular cholesterol was found in the top 0.4 ml of the 5-ml centrifuge tube, as assessed by gel filtration chromatography. This fraction contained <8% of cholesterol carried in micelles, as assessed by gel filtration chromatography. The variation coefficient of this short ultracentrifugal method to isolate biliary vesicles was 4.6%. The frequency values of vesicles' contribution to biliary cholesterol transport by this ultracentrifugal method are comparable to those reported previously by other investigators using gel filtration chromatography and elution buffers containing bile acids in different concentrations. The procedure reported in this study is simple, accurate, and fast for physiologic, pathophysiologic, and clinical studies. —**Amigo, L., C. Covarrubias, and F. Nervi.** Rapid isolation of vesicular and micellar carriers of biliary lipids by ultracentrifugation. *J. Lipid Res.* 1990. **31:** 341–347.

Supplementary key words vertical rotor • biliary vesicles • micelles • Metrizamide

For several decades it was generally accepted that cholesterol in native bile was solubilized in bile salt-phospholipid-mixed micelles similar to those obtained in model bile solutions (1–3). This concept was the basis for both the micellar theory of biliary lipid secretion and the pathophysiological model of cholesterol gallstone formation (4, 5).

In the last few years various groups have clearly demonstrated the presence of vesicles in both native human supersaturated and rat unsaturated bile (6–8). This carrier is responsible for the transport of a significant amount of biliary cholesterol. This changing concept of cholesterol transport in bile has had a major significance for the better understanding of biliary lipid secretion from the hepatocyte and nucleation, the critical phase of cholesterol gallstone formation.

Two techniques are currently used to separate vesicles from micelles in bile: gel filtration chromatography and density gradient ultracentrifugation (6–8). The major shortcoming of gel filtration chromatography to quantitate the proportion of vesicles and micelles in bile derives from the critical fact that bile specimens must be exposed to large volumes of elution buffers containing bile salts in variable concentrations. The presence or absence of bile salts in the elution buffers drastically changes the proportion of vesicles and micelles in bile (9). It is also theoretically possible that ultracentrifugation alters the proportion of vesicles and micelles. Artefacts may result because of chemical interactions between vesicles, micelles, and the centrifugation media used for isopycnic centrifugation of the bile samples, or because of the hydrostatic pressure generated by the centrifugal force in the tubes, which may reach 1,500 kg/cm^2 in the commonly used fixed angle rotors. This high hydrostatic pressure may destroy vesicles as happens with intracellular membranes. In addition, the centrifugation time required to separate vesicles from micelles in bile specimens using fixed-angle rotors has ranged from 19 to 65 h (6–8). This time may be sufficient to dissolve a significant proportion of vesicular lipids into mixed micelles (8).

The purpose of this study is to describe and validate a rapid ultracentrifugal method to separate vesicles from micelles and to quantitate them in bile specimens. We used a vertical rotor to markedly reduce the centrifugation time

and Metrizamide, which is one of the best inert gradient media, as the centrifugation medium. It has proved suitable for the separation of almost all biological particles (10).

MATERIALS AND METHODS

Bile specimens

Fresh hepatic bile was obtained from several patients at least 1 week after choledochotomy with an open indwelling T-tube. All patients had common duct stones. All bile specimens (approximately 20 ml) were collected at ambient temperature (20° to 25°C) and immediately transported to the laboratory. The age range of the patients was 33 to 70 years.

The samples were centrifuged at 10,000 rpm for 10 min at 10°C in a Sorvall centrifuge (DuPont Instruments) and then filtered through a 0.8 to 8 μm pore size (type AP 20 Millipore) prefilter.

Ultracentrifugation

The density of the bile samples was modified with Metrizamide [2-(3-acetamido-5-N-methylacetamido-2,4,6,triiodobenzamido)-2-deoxy-D-glucose] (Sigma Chemical Co., St. Louis, MO) as previously described (8). Metrizamide was directly dissolved in bile (18% w/v). In some experiments, continuous gradients were prepared with pure bile mixed with 18% Metrizamide in bile by means of a Bechman density gradient former (Beckman Instruments, Inc., Palo Alto, CA). The density limits of the gradients were between 1.009 and 1.150 g/ml. The gradients were prepared in 5-ml Quick-Seal tubes (Beckman Instruments. Inc.) and were centrifuged at 50,000 rpm for 285 min at 10°C in a VTi 65 vertical rotor (Beckman Instruments, Inc.). Seven fractions were collected by tube puncturing and weighed in a tared tube. Bile salts, phospholipids, and cholesterol were determined in each fraction. The fraction densitites were measured according to the method of Beaufay et al. (11). Briefly, a small aliquot (10 to 15 μl) of the fraction was allowed to settle in a cold column thermostatically controlled with a gradient of 1,2-dichlorobenzene and petroleum ether (boiling point, 80°- 100°C). The gradient was calibrated with KBr solutions of known densities. Ultramicroscopy of the fractions was performed to confirm the presence of vesicles as previously described (8). In some experiments, 0.25 M sucrose or 16% Metrizamide were directly dissolved in bile for isolation of vesicles in the top 0.4 ml of the 5-ml Quick-Seal tube after 120 min of centrifugation at 50,000 rpm at 10°C in the VTi-65 vertical rotor.

Gel filtration chromatography

Gel filtration chromatographic analysis of bile-Metrizamide density fraction and pure bile (0.2–0.4 ml samples) were done on Ultrogel AcA 34 (Pharmacia columns) (310 × 8 mm) with a flow rate of 0.5 ml/min. Blue Dextran, (Sigma Chemical Co.) mol wt 2×10^6, was used as a marker for the void volume and [^{14}C]sucrose (Amersham, Buckinghamshire, England) was used to detect the end of the elution from the columns. The exclusion volume of the columns varied between 7 and 9 ml. The elution buffer contained 50 mM Tris (hydroxy-methyl-aminomethane) (Sigma Chemical Co.), 1.5 mM Tritriplex III, and 150 mM NaCl, pH 8. The buffer also contained taurocholate (Sigma Chemical Co.) in variable concentrations according to the total bile salt concentration previously determined in bile and/or fractions collected after centrifugation. In this manner, we intended to preserve the proportion of vesicles and micelles originally present in the specimens. Fractions (1 ml each) were collected for determination of radioactive cholesterol.

Chemical analysis

Biliary lipids were quantitated as previously described (8). In addition, to rapidly determine the vesicular fraction in the chromatographic studies, bile specimens were incubated with [^{3}H]cholesterol (Amersham). Radioactive cholesterol in toluene, approximately 0.25 μCi, was added to a glass tube and evaporated under N_2 at 37°C. Bile specimens were then added to the tubes and incubated for 1 h at room temperature with gentle shaking. Correlation coefficients between cholesterol chemically determined and cholesterol concentration calculated by radioactivity analysis were better than 0.97 in a series of 12 determinations.

RESULTS

Distribution of biliary lipids in bile-Metrizamide density gradients

The concentrations of bile salts, phospholipids, cholesterol, the cholesterol to phospholipid ratio, and the relative distribution of each biliary lipid in the different fractions of a typical bile-Metrizamide density gradient as a function of the density of the gradient are shown in **Table 1**. The mean density of fractions of this gradient varied between 1.018 and 1.123 g/ml. It is noteworthy that there is a striking dissociation between biliary bile salts and cholesterol; approximately 70% of total biliary cholesterol was harvested in fractions with densities < 1.030 g/ml. In contrast, 70% of bile salts was found in the fractions with densities > 1.030 g/ml. Electron microscopy demonstrated the presence of numerous vesicles in the first three fractions of the gradient d < 1.030 g/ml). The number of vesicles progressively decreased in fractions with higher densities. The mean diameter of vesicles in the d 1.018 g/ml was 82 $\pm$10 nm (mean $\pm$ SD) in a series of 24 vesicles. In contrast, the diameter of vesicles harvested in

TABLE 1. Biliary lipid concentrations and cholesterol/phospholipid ratios in bile-Metrizamide fractions obtained from hepatic bile

Fraction	Density of Fraction	Volume of Fractions	Biliary Lipid Concentration			% Biliary lipid in Each Fraction			Cholesterol / Phospholipid
			Bile Salt	Phospholipid	Cholesterol	Bile Salt	Phospholipid	Cholesterol	
	g/ml	*ml*		*mM*			%		
1	1.018	0.56	26	10.0	12.2	10.5	14.6	41.4	1.22
2	1.024	0.69	19	6.6	5.8	9.5	12.1	24.1	0.87
3	1.030	0.73	17	5.0	1.8	9.1	9.6	7.9	0.36
4	1.044	0.64	23	6.7	1.9	10.5	11.4	6.9	0.26
5	1.058	0.65	28	7.5	1.5	13.1	12.8	6.0	0.21
6	1.074	0.70	32	8.8	1.3	16.1	16.3	5.7	0.15
7	1.123	0.84	52	10.5	1.6	31.2	23.2	8.0	0.15
Whole bile			25.5	8.0	3.7	100	100	100	0.46

Bile-Metrizamide continuous density gradient was prepared within 1 h of bile collection. Metrizamide was directly dissolved in bile (18% w/v). The gradient was centrifuged at 50,000 rpm for 285 min in a VTi 65 vertical rotor (Beckman Instruments, Inc.). Bile fractions were collected by tube puncture. Hepatic bile was obtained at 8 AM from a patient 1 week after choledochotomy for common duct stones.

the d 1.030 g/ml fraction was 57 ± 8 nm in a series of 24 vesicles. The difference of the vesicle diameters of both fractions was significant at the $P < 0.001$ level. This finding indicates that the population of biliary vesicles is heterogeneous in size. **Table 2** shows the percent contribution of vesicles and micelles in a series of six fresh bile-Metrizamide density gradients. Each fraction of the gradient was applied to the Ultrogel AcA 34 chromatographic columns and eluted with a buffer containing taurocholate at the same concentrations found in the specific fraction. In this manner, we could calculate that 65 ± 13% of total biliary cholesterol was carried in vesicles, whereas 35 ± 13% was carried in micelles. Approximately 93% of total vesicular carriers was found in the d < 1.030 g/ml fraction.

In addition, after column chromatography of each fraction it could be calculated that 93 ± 4% of cholesterol present in the d < 1.030 g/ml fraction was solubilized in vesicles and only 7 ± 4% in micelles. The effect of the potential artefact induced by inert centrifugation media such as Metrizamide and sucrose on the proportion of vesicles and micelles present in native bile was evaluated by gel filtration chromatography as shown in **Fig. 1**. It can be seen that neither Metrizamide nor sucrose disturbed the original distribution of lipid carriers present in the bile.

Rapid ultracentrifugal isolation of vesicles from native bile

In the next series of experiments we attempted to isolate vesicles from native bile using 16% Metrizamide directly dissolved in bile without gradient formation. This concentration of Metrizamide increased the density of bile to approximately

TABLE 2. Biliary lipid composition and percent vesicular and micellar cholesterol found in density < 1.030 g/ml fractions of hepatic bile

Bile Specimen	Biliary Lipid Concentration			% Biliary Cholesterol in		% Total Vesicular Cholesterol in d < 1.030 Fractions	Cholesterol / Phospholipid in d < 1.030 Fractions	% Cholesterol in d < 1.030 Fractions	
	Bile Salt	Phospholipid	Cholesterol	V	M			V	M
		mM		%		%		%	
1	24	6.5	7.9	71	29	92	0.52	91	9
2	15	5.0	1.8	73	27	87	0.74	97	3
3	47	10.3	3.5	44	56	98	1.00	85	15
4	13	4.0	1.9	61	39	88	0.90	95	5
5	32	6.0	1.9	59	41	92	1.00	95	5
6	20	5.2	3.7	82	18	99	0.90	95	5
Mean ± SD	25 ± 13	6 ± 2	3.5 ± 2	65 ± 13	35 ± 13	93 ± 5	0.85 ± 0.2	93 ± 4	7 ± 4

Bile-Metrizamide density gradients were formed as described in Table 1. After ultracentrifugation, aliquots of each fraction of the gradient were applied to Ultrogel AcA 34 columns, 0.8 cm diameter and 31 cm long. The elution buffer of each column contained taurocholate according to the bile salt concentration previously found in each fraction of the gradient; V, vesicles; M, micelles.

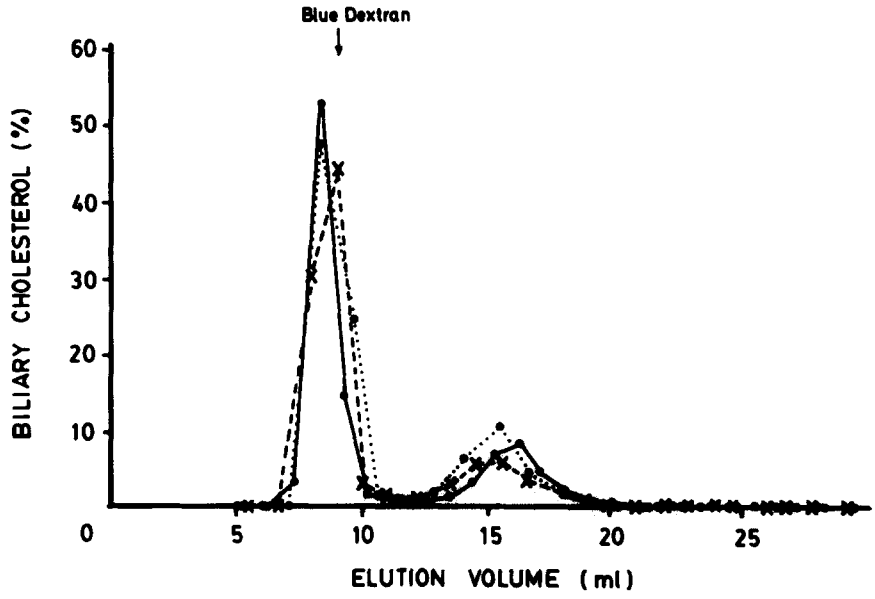

Fig. 1. Effect of 16% Metrizamide and 0.25 M sucrose on the proportion of vesicular and micellar carriers of biliary lipids in native bile. Pure bile (●—●), bile containing 16% Metrizamide (x---x), or 0.25 M sucrose (○· · ·○) were subjected to gel filtration chromatography as described in Table 2. The void volume marker was Blue Dextran. The first peak represents the vesicular fraction, corresponding with the exclusion volume of the column, and the second peak corresponds to the micellar fraction. The elution buffer contained 50 mM Tris, 150 mM Tritriplex III, pH 8, and 10 mM Na cholate.

1.060 g/ml. Vesicles were separated after 2 h of centrifugation in a series of 12 bile specimens as shown in **Fig. 2** and **Table 3**. It was found that the upper 0.4 ml of the centrifuge tube presented a white opalescent layer, whereas the lower 4.6 ml appeared isotropic. Both fractions where then subjected to chromatographic analysis as shown in Fig. 2. It was clearly found that the upper layer was excluded from the column indicating that this fraction contained vesicles. In contrast, the lower phase was included in the column, indicating the presence of a polymolecular complex compatible with mixed micelles. Approximately 62% of total biliary cholesterol of native hepatic bile was dissolved in vesicles in a series of 12 consecutive experiments, as shown in Table 3. Approximately 93% of cholesterol present in the upper 0.4 ml of the centrifuge tube was dissolved in vesicles. It was also found that more than 97% of total vesicular cholesterol was harvested in the upper 0.4-ml layer of the centrifuge tube, as assessed by gel filtration chromatography. The variation coefficient of this ultracentrifugal method was 4.6% in a series of six replicate analyses.

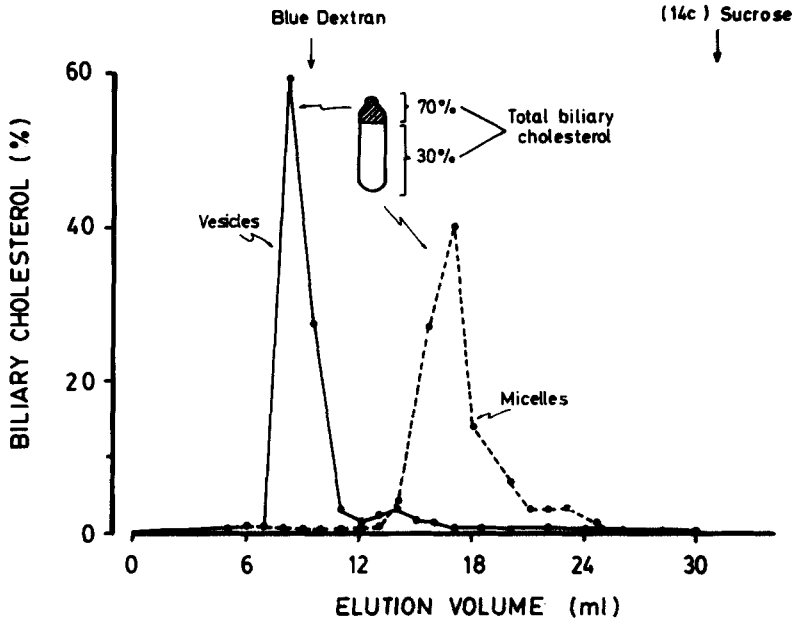

Fig. 2. Gel filtration chromatography of the upper 0.4 ml (opalescent, represented by the hatched area) and the lower 4.6 ml (isotropic bile, represented by the white portion) of the centrifuge tube. Native hepatic bile containing 16% Metrizamide was centrifuged in a vertical VTi 65 rotor at 50,000 rpm for 2 h. After centrifugation the upper and lower fractions were subjected to gel filtration chromatography as described in Table 2. The continuous line represents the upper 0.4 ml and the discontinuous line the lower 4.6 ml which contain the micellar carrier of biliary cholesterol.

TABLE 3. Biliary lipid composition and percent vesicular and micellar cholesterol isolated by a short-run ultracentrifugation from hepatic bile

Bile Specimen	Biliary Lipid Concentration			% Biliary Cholesterol in		Cholesterol / Phospholipid	
	Bile Salt	Phospholipid	Cholesterol	V	M	V	M
		mM			%		
1	16	8.2	3.6	76	24	0.82	0.14
2	28	7.8	2.2	48	52	0.94	0.21
3	8	3.1	1.4	72	28	0.97	0.21
4	5	2.1	1.1	77	23	0.91	0.34
5	25	13.2	4.7	45	55	0.95	0.22
6	17	5.5	1.9	70	30	1.06	0.18
7	29	9.8	5.8	65	35	1.26	0.24
8	27	9.2	3.2	56	44	0.98	0.17
9	21	8.0	3.7	50	50	1.22	0.21
10	19	11.5	6.9	61	39	1.06	0.25
11	26	11.9	4.3	48	52	0.96	0.20
12	10	3.1	1.4	71	29	0.96	0.19
Mean ± SD	19 ± 8	7.8 ± 3.7	3.4 ± 1.9	62 ± 12	38 ± 12	1.01 ± 0.13	0.21 ± 0.05

Fresh hepatic bile was obtained from 12 patients 5 to 7 days after surgery. Bile with 16% Metrizamide was centrifuged at 50,000 rpm for 2 h in a VTi-65 vertical rotor. The upper and lower phases were chromatographed as described in Table 2. More than 96% of total vesicular cholesterol was harvested in the upper 0.4 ml of the centrifuge tube. Micellar cholesterol represented < 8% of total cholesterol harvested in the upper layer; V, represents the upper 0.4 ml; M, represents the lower 4.6 ml of the tube.

A comparison of this rapid ultracentrifugal method with gel filtration chromatography using 10 mM Na cholate is shown in **Table 4**. It can be seen that the percent vesicular cholesterol estimated by chromatography was slightly higher in five of six different bile specimens compared with the ultracentrifugal method. However, the mean values were not significantly different.

TABLE 4. Contribution of vesicular carriers to biliary cholesterol transport in vesicles isolated by ultracentrifugation and gel filtration chromatography

Bile Salt Concentration	Percent Biliary Cholesterol in Vesicles Isolated by	
	Ultracentrifugation	Chromatography
mM	%	
29	59	66
21	40	54
19	60	71
27	51	47
25	40	50
26	43	49
25 ± 3	49 ± 9	56 ± 10

Fresh hepatic bile was obtained from six patients 5 to 7 days after surgery. Bile with 16% Metrizamide was centrifuged at 50,000 rpm for 2 h in a VTi-65 vertical rotor. The upper and lower phases were chromatographed as described in Table 2. A bile specimen was also directly subjected to gel filtration chromatography in Ultrogel AcA 34 columns, 0.8 cm diameter and 31 cm long. The elution buffer in this case contained 10 mM Na cholate.

In the final series of experiments we evaluated the effects of temperaure of centrifugation, time of storing the bile specimens prior to separating the biliary lipid carriers, and the presence of different Na cholate concentrations in the centrifuge tube. The objective of this experiment was to evaluate the ultracentrifugal method to isolate vesicles from small bile specimens layered on a buffer containing 16% Metrizamide and Na cholate. **Fig. 3** shows that the proportion of vesicles and micelles did not change significantly whether we set the temperature of centrifugation at 4°C or at 24°C. Similarly, it can be seen that the proportion of vesicles and micelles was kept constant as a function of time between 2 and 24 h after bile collection. **Fig. 4** shows that the percent of biliary cholesterol carried in vesicles markedly changed as a function of Na cholate concentration used for the 4 ml of buffer present in the centrifuge tubes. As expected, the proportion of vesicles was reciprocally related to the Na cholate between 20 mM and absence of the bile acid in the buffer.

DISCUSSION

This series of experiments demonstrates that the vesicular carrier of biliary lipids may be rapidly isolated, characterized in terms of composition, and quantitated by isopycnic centrifugation using a vertical rotor. Vesicles are concentrated in densities < 1.030 g/ml with less than 10% of vesicles isolated in densities between 1.030 and 1.060

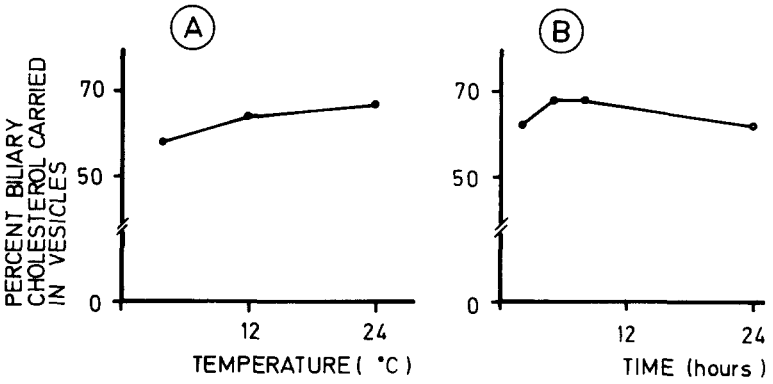

Fig. 3. Effect of temperature of centrifugation (panel A) and time after bile collection on the percentage of biliary cholesterol carried in vesicles separated by ultracentrifugation as described in Table 3. The temperature was maintained at 4°C throughout the 24-h period in a cold room in the experiments shown in panel B. The cholesterol/phospholipid ratio of the vesicular fraction remained constant during the 24 h of observation. Similarly, it was not modified by the temperature of centrifugation.

g/ml. It is apparent from the experiments and from a previous study (8) that the population of vesicles is heterogeneous in size as determined by electron microscopy. Larger particles with a mean diameter of 80 nm have a cholesterol/phospholipid ratio of approximately 1.2, whereas vesicles isolated in d 1.030 g/ml fractions have a mean diameter of 57 nm and a cholesterol/phospholipid ratio of approximately 0.85. The physiological significance of this difference is unknown. The mean diameters of vesicles reported in this study are in the range found by electron microscopy after freeze- fracture and quasielastic light scattering (12).

Although the quantitative contribution of vesicles and micelles to biliary cholesterol transport estimated by the present method is in the range of the values obtained with gel filtration chromatography using 10 mM Na cholate as elution buffer (6, 9), this ultracentrifugal method has two major advantages. First, vesicles can be isolated in a reproducible manner in 150 min, a time that is approximately half the time required to perform a chromatographic separation. Second, vesicles can be harvested from the original bile specimen without the addition of exogenous bile salts or the dilutional effect represented by the volume of elution buffers used for column chromatography. In this manner, the ultracentrifugal method allows the isolation of vesicles for a relatively more accurate determination of their composition. It is important to emphasize, however, that all methods presently used to separate biliary lipid carriers, including ultracentrifugation and gel filtration chromatography, give only a relative estimation of the proportion of vesicles and micelles in bile.

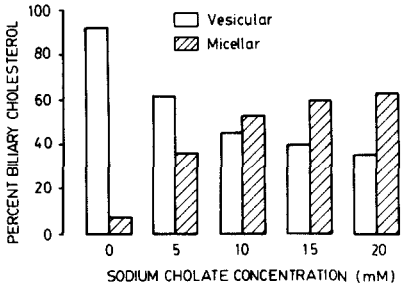

Fig. 4. Effect of different Na cholate concentrations in the lower bed of a centrifuge tube on the porportion of vesicles and micelles present in small aliquots of native bile. One ml of hepatic bile containing 7% Metrizamide was layered on 4 ml of buffer containing 50 mM Tris, 150 mM NaCl, and 1.5 mM Titriplex III at pH 8. Different concentrations of Na cholate and 16% Metrizamide were added to the buffer. After centrifugation as described in Table 3, aliquots of the upper 0.5 ml and lower 4.5 ml were subjected to gel filtration chromatography with the same buffer used for the discontinuous gradient.

The potential pitfalls of the ultracentrifugal method described in this study are related to the osmotic effects of the gradient media and the hydrostatic pressure generated in the centrifuge tube. Although both sucrose and Metrizamide are inert centrifugation media, Metrizamide has two major advantages over sucrose. First, it allows the spontaneous formation of density gradients during centrifugation and second, Metrizamide has a much lower osmotic effect as compared with sucrose (13, 14). This method called isopycnic centrifugation, is an equlibrium method, yielding a series of zones of particles each banded at its characteristic buoyant density (13). In this manner, different populations of vesicles can be harvested from the original bile. Osmotic effects induced by the centrifugation media on the size of vesicles or other cellular organelles are largely reduced by Metrizamide as compared with sucrose (14). In fact, Metrizamide gradients allow the banding of cellular organelles in gradients that are isotonic throughout, thus obviating any damage due to osmotic swelling or shrinking (15). It is known that when solutions or suspensions are centrifuged, hydrostatic pressure is generated in the column represented by the centrifuge tube (15). This effect is separate from that of osmotic stress and it occurs even in gradients that are isotonic throughout (16). The pressure generated is considerable at high speeds reaching values of 1,000 to 1,500 g/cm^2 in fixed-angle rotors (16, 17). This potential damaging effect on the vesicular carrier of biliary lipids may be markedly reduced by at least one order of magnitude using a vertical rotor. This rotor has the advantages of a shorter radius and a hydrostatic column equivalent to approximately one-

fifth of the hydrostatic column of the fixed-angle rotors. It is very likely, then, the stress of compression on the vesicular and micellar structures, is markedly reduced with the present ultracentrifugal method, which allows both a reduction of time of centrifugation and of pressure in the hydrostatic column by approximately a factor of ten.

An important observation of the present study relates to the stability of biliary vesicles. We have shown that the proportion of vesicles in bile remains constant whether centrifuged at 4°C or at 24°C. In addition, freezing and thawing once does not significantly alter the proportion of vesicles and micelles of native human bile, but markedly reduces the proportion of vesicles in unsaturated rat bile (Amigo, L., C. Covarrubias, and F. Nervi, unpublished observations). It is apparent that freshly collected T-tube native bile has already reached an equilibrium since the proportion of vesicles and micelles remains constant, at least over 24 h of observation, as shown in the present study.

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