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Effects of Freezing and Incubation on Biliary Lipid Analysis (38276)

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A number of physical factors may affect the relative concentrations of the biliary lipids found in human bile. These factors include stasis, temperature, oral antibiotics, and the presence of bacteria (1). The methods used for handling and storing bile which has been collected for analysis vary considerably from one report to another. These methods include immediate analysis (2), millipore filtration (3), refrigeration (4), slow or quick freezing with analysis at a later date (5–7), and incubation in a 37° shaking water bath (8). The literature does not contain clear data as to what alterations, if any, are brought about by these various physical manipulations.

Methods. The gallbladders of eight patients without gallstones were completely aspirated through a 20-gauge needle after temporary occlusion of the cystic duct. All patients had normal gallbladder function and were undergoing elective surgery. No patient received antibiotics. The bile was obtained within 45 min following the induction of anesthesia and transported immediately to the laboratory where the pH was determined. The bile was then cultured and centrifuged at 3000 rpm for 20 min. The sediment was examined under polarized light for cholesterol crystals and the supernatant was divided into three equal aliquots — fresh, incubated, and fast frozen. Incubation was carried out in a shaking water bath at 37° after first flushing the sealed, sterilized tube of bile with nitrogen. Fast freezing was carried out by immersion of the sealed aliquot of bile into liquid nitrogen (−170°) followed by storage at −30°. Cholesterol, phospholipid, and bile acid determinations were performed immediately on the fresh bile and at the end of 5 days on the incubated and fast-frozen bile. The cultures and pH determinations on the incubated bile were repeated at 5 days. All of the biliary lipid determinations were carried out at room temperature using the methods previously described (9). The remaining portions of the fesh, incubated, and fast-frozen bile samples were passed through a 0.22-μm millipore filter and the cholesterol, phospholipid, and bile acid determinations were repeated on these filtered aliquots.

Statistical comparisons were made using the Student's t test for paired variables with $P < 0.05$ taken as the level of significance.

Results. All cultures were negative for bacterial growth. Cholesterol crystals were present in the sediment of fresh centrifuged bile in 3 of the 8 patients and in all of the fast-frozen aliquots.

The biliary lipids of each of the eight patients analyzed in the fresh state were not significantly altered by millipore filtration (Table I). Similarly, filtration did not significantly alter the biliary lipids in those bile samples which were incubated for 5 days prior to analysis (Table II). Filtration did, however, lower biliary cholesterol from 803 ± 102 to 706 ± 75 mg% ($P < 0.025$) in those bile samples which were fast frozen and stored for 5 days prior to analysis although phospholipids and bile acids were not significantly altered in this group (Table III).

Additional comparisons were made to determine the effects of incubation and fast freezing on bile composition (Table IV). Phospholipid concentration fell from 3978 ± 399 mg% in fresh bile to 3747 ± 389 mg% in incubated bile ($P < .005$). A significant fall in concentration

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TABLE I. Biliary Lipids (mg%) of 8 Patients Without Gallstones Analyzed in the Fresh and Fresh Filtered States.

	Cholesterol		Phospholipids		Bile acids	
Patient	Fresh	Filtered	Fresh	Filtered	Fresh	Filtered
1	743	755	3823	3899	160	169
2	758	764	3078	3071	131	131
3	1385	1352	6105	6042	191	193
4	722	714	4682	4758	221	220
5	427	428	2951	2958	176	176
6	787	784	3442	3442	204	200
7	836	861	4799	4735	232	224
8	623	611	2947	2952	143	140
$\bar{X}\pm$SE	785±97	784±94	3978±399	3982±393	182±13	181±12
P	> 1		> .1		> 0.1	

was also measured when we compared the phospholipid levels in fresh filtered bile (3982 ± 393 mg%) to incubated filtered bile (3765 ± 389 mg%). Fast freezing did not affect phospholipid concentration but the concentration of cholesterol rose from 785 ± 97 mg% in fresh bile to 803 ± 102 mg% in the fast-frozen bile ($P < .01$). Millipore filtration lowered the cholesterol concentration in fast-frozen bile from 803 ± 102 to 706 ± 75 mg% ($P < .025$). This level of statistical significance was sustained when the cholesterol concentration of fresh filtered bile (784 ± 94 mg%) was compared to that in the fast-frozen bile (706 ± 75 mg%).

Discussion. Various indices are used to measure the lithogenic potential of bile (2–4). These indices depend upon the concentrations of bile acids and phospholipids in relation to cholesterol. Any physical alteration of bile may change the relative amounts of the three biliary lipids, resulting in data from the analysis which are spurious and which describe a bile of different composition and lithogenicity. There does not appear to be any methodologic uniformity followed when bile is collected for analysis. Many authors freeze or refrigerate the bile which is then stored until a suitable number of specimens have been collected (6, 7, 10, 11), whereas others analyze bile as soon as it has been collected (2). Millipore filtration is considered essential for accurate phase separation of bile by some investigators (8, 12) and the determination of cholesterol solubilizing capacity, as proposed by others, requires incubation at 37° in a shaking water bath (13, 18).

The significant reduction of phospholipids in the incubated compared to the freshly analyzed

TABLE II. Biliary Lipids (mg%) of 8 Patients Without Gallstones Analyzed in the Incubated and Incubated Filtered States.

	Cholesterol		Phospholipids		Bile acids	
Patient	Incubated	Filtered	Incubated	Filtered	Incubated	Filtered
1	753	747	3290	3304	165	161
2	631	567	2709	2798	129	126
3	1344	1331	5847	5897	184	199
4	712	719	4389	4453	233	230
5	417	421	3033	2962	184	181
6	769	725	3301	3271	207	199
7	884	889	4620	4563	235	233
8	629	614	2794	2872	139	141
$\bar{X}\pm$SE	767±95	752±96	3748±389	3765±389	184±14	184±14
P	> 0.05		> 0.1		> 0.1	

TABLE III. Biliary Lipids (mg%) of 8 Patients Without Gallstones Analyzed in the Fast-Frozen and Fast-Frozen-Filtered State.

	Cholesterol		Phospholipids		Bile acids	
Patient	Fast Frozen	Filtered	Fast Frozen	Filtered	Fast Frozen	Filtered
1	769	660	3733	3695	165	161
2	774	577	3126	2862	124	126
3	1437	1120	6419	6037	189	195
4	730	687	4507	4682	221	221
5	428	419	3105	3028	178	189
6	804	787	3569	3636	208	201
7	853	825	4614	4723	229	223
8	629	572	2977	2834	139	142
$\bar{X}\pm$SE	803±102	706±75	2013±410	3937±399	182±13	182±13
P	> 0.025		> 0.01		> 0.1	

bile may have been due to the conversion of lecithin to lysolecithin and fatty acids (14) although for this to occur there must be contamination by either bacteria or pancreatic secretions (18). This seems unlikely since all cultures were negative and because the gallbladder was aspirated only after occlusion of the cystic duct.

Cholesterol is totally insoluble in water. In order for micelles to form, the bile must be above its critical micellar temperature—the Kraft Point—whereas below this temperature, crystals may form (13). Bile which was fast frozen and subsequently filtered contained significantly lower cholesterol concentrations because of the formation of cholesterol crystals which did not pass through the millipore. These filters contained large numbers of cholesterol crystals, whereas the millipores used for filtering the fresh bile were virtually free of crystals. Although not infrequently found in normal bile, the presence of cholesterol crystals, especially in clumps, is generally thought to indicate that the bile is either lithogenic or else contains gallstones.

We did not investigate the effects of slow freezing at $-20°$ since it has already been shown that when this method is used cholesterol is precipitated and removed by millipore filtration (16, 17). It is apparent then that none of fast freezing, slow freezing, or incubation are without effects on the composition of gallbladder bile. Incubation lowers phospholipids while freezing results in crystallization which may spuriously elevate the cholesterol levels of unfiltered bile and which reduces measured cholesterol when the bile is filtered.

Normal human bile is nearly saturated with

TABLE IV. Effects of Different Methods of Storing Bile on Biliary Lipids (mg%) in the Gallbladder Bile of 8 Patients Without Gallstones. Results Expressed as Mean (± SE).

Comparison	Cholesterol	Phospholipids	Bile acids
Fresh	785(± 97)	3978(± 399)[b]	182(± 13)
Incubated	767(± 95)	3747(± 389)	184(± 14)
Fresh filtered	752(± 94)	3982(± 393)[b]	181(± 12)
Incubated filtered	752(± 96)	3765(± 389)	184(± 14)
Fresh	785(± 97)[a]	3978(± 399)	182(± 13)
Fast frozen	803(± 102)	4014(± 410)	182(± 13)
Fresh filtered	784(± 94)[a]	3982(± 393)	181(± 12)
Fast frozen filtered	706(± 75)	3937(± 399)	182(± 13)

[a] $P < .005$.
[b] $P < .01$.

cholesterol. Since the maintenance of biliary cholesterol in micellar solution is dependent upon the relative proportions of cholesterol, bile salts, and phospholipids, a small increase in cholesterol or a decrease in phospholipids and/or bile salts could produce a bile which is unstable with respect to cholesterol solubility. Currently, there is controversy over both the limits of cholesterol solubility as well as the significance of supersaturated bile (8, 19). The present study was designed to examine the effects of handling and storage on biliary lipid composition in an attempt to determine whether or not the aforementioned methodologic variations could explain, at least in part, the conflicting reports on the solubility limits of cholesterol in bile. Our data strongly suggest that bile should be analyzed in the fresh state. Filtration is useful in removing solid material from the bile and does not significantly alter the biliary lipid composition of fresh bile. If analysis in the fesh state is not possible, it would seem appropriate to freeze the *exact* volume of bile required. The various chemical determinations could then be done on the entire volume of the aliquot after thawing.

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