Table 1: Methanol, fusel oil and ethanol contents of beers

Sample	% Methanol (v/v)	Fusel Oil (ppm)	% Ethanol (v/v)
1. chibuku – village	0.013	150	3.6
2. chibuku – village	0.016	140	5.2
3. chibuku – village	0.016	168	4.7
4. chibuku – commercial	0	80	3.0
5. chibuku – commercial	0	144	2.3
6. chibuku – laboratory	trace	80	2.3
7. sugar beer – laboratory	0	26	3.4
8. ginger beer	0.070	152	5.2

Table 2: Methanol, fusel oil and ethanol contents of distilled drinks.

Sample	% Methanol (v/v)	Fusel Oil (ppm)	% Ethanol (v/v)
1. kachasu – maize	0.107	411	14.7
2. kachasu – maize	0.130	234	18.7
3. kachasu – maize (kachibembe)	0.100	250	20.2
4. kachasu – cassava	0.170	242	13.0 ●

Biliary Lipids and the paucity of Cholesterol Gallstone Disease in Zambia

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INTRODUCTION

The concept that cholesterol gallstone formation is the result of precipitation of cholesterol from super-saturated bile has been confirmed by the demonstration that gallstone-containing bladder bile had a concentration of cholesterol at or near the limit of its solubility (Admirant and Small, 1968). Apparently closing a prolonged debate as to whether the primary defect was in the hepatic cell or in the gallbladder, the demonstration that *hepatic* bile from gallstone patients is saturated or supersaturated with cholesterol has led to the widely held view that cholesterol gallstone disease results from a derangement of liver metabolism (Vlahcevic *et al*, 1970). Some workers consider that this derangement of metabolism is due to an external factor such as diet. The evidence implicates the Western type of diet comprising largely of refined foods and in particular sucrose. Cholesterol gallstone disease has become increasingly common among the Japanese, for example, since their introduction of a Western way of life.

Other workers believe the metabolic derangement is due not so much to those constituents present in refined foods, but to their reduced content of dietary fibre (Trowell, 1972). As dietary fibre binds bile salts in the intestine, it may be a factor of prime importance in lowering serum cholesterol and the incidence of gallstone disease in populations consuming largely cereal diets.

The present investigation is concerned with biliary lipid levels in normal Zambian subjects, amongst whom gallstone disease is exceedingly rare. The results suggest an alternative pathogenesis for cholesterol gallstone disease and also provide a baseline for future investigations in connection with any trend away from traditional dietary habits.

METHODS

Gallbladder Bile

Postmortem specimens of gallbladder bile were collected from 15 Zambian subjects (10 male, 5 female) who had died as a result of trauma but otherwise showed no disease. Three specimens of Caucasian gallbladder bile and one of hepatic bile obtained during cholecystectomy were also analysed.

Analytical Methods

Analyses were performed for total bile acids, phospholipids and cholesterol.

Total bile acids were measured by the hydroxysteroid dehydrogenase method (Turnberg and Anthony-Mote, 1968: Murphy *et al*, 1970). Bile (0.25ml) was diluted to 5.0ml with distilled water and centrifuged. Supernatant (4.0ml) was mixed with methanol-acetone (12ml, 1:1, v/v) and centrifuged again. The supernatant was collected, the residue washed with methanol-acetone (4.0ml) and the supernatants combined. The extracts were evaporated to dryness and redissolved in aqueous ethanol (3.0ml, 50%). An aliquot (20ul) of this extract was placed in a cuvette and the following reagents added in sequence: — i. Sodium pyrophosphate solution (1.1ml, 0.1 M, previously adjusted to pH 10.8 with 0.1 N NaOH), ii. Hydrazine hydrate solution (1.0ml, prepared from 5.0 ml hydrazine hydrate in ice cold water by adding 1.5ml 2N H_2SO_4 and diluting to 100ml) iii. NAD solution (0.5ml, 41mg NAD dissolved in 10ml water and pH adjusted to 7.0 with $NaHCO_3$), iv. Enzyme solution (0.1 ml, 10 mg/ml, Grade II, Sigma Chemicals Co.). The extinction at 340 nm was measured after 30 minutes. A blank consisted of the above mixture but with 20ul of aq. ethanol in place of extract.

Phospholipids were analysed by perchloric acid digestion of whole bile followed by the estimation of inorganic phosphorus (Connerty *et al*, 1961). Bile (0.01ml) was digested with a mixture of perchloric acid (70%) — H_2SO_4 — water (1.0ml, 1:1 v/v) until colourless. After cooling, water (1.0ml) was added and the solution boiled for 15 seconds Sodium acetate (1.0ml, 50%) was added then the whole made up to 10ml with water. Ammonium molybdate (1.0ml, 2.5%) and Metol (1.0ml, 1.0%) were added, then after 15 minutes the extinction was measured at 700 nm. A blank comprised the the above reagents with omission of the bile. A standard consisted of:— working standard (5.0ml, a 1 in 250 dilution of stock standard comprising 4.394g KH_2PO_4 per litre of solution containing 2.0ml H_2SO_4 with H_2SO_4 (0.25ml), sodium acetate solution (1.0ml), ammonium molybdate solution (1.0ml), Metol solution (1.0ml) and water (3.75ml).

Cholesterol was measured by a modified Lieberman-Burchard reagent (Rappaport and Eichorn, 1955) after prior chromatographic separation from bile. Bile (0.2ml) was added dropwise to alumina (3.5g, Grade I for chromatography) and well mixed. This was then placed on a pre-prepared column of alumina (1.9g, diam. 0.8cm) and the cholesterol eluted with chloroform-methanol (95:5 v/v). A volume of 5.0ml of eluate was collected and evaporated to dryness. Sulphosalicylic acid reagent was added (2.0ml, 12% in glacial acetic acid), and tubes swirled and acetic anhydride (5.0ml) added. After cooling at 25^o for 10 minutes, H2SO4 (0.7ml) was added then the solution mixed well and stood in the dark at 25^o for 15 minutes. The extinction at 600 nm was then read against the appropriate blank. A blank was prepared for each eluate, consisting of a second eluate dissolved in 7.7ml of aq. acetic acid (25%). Cholesterol standards containing 100, 200 and 300 mg per 100ml were prepared in glacial acetic acid and used to construct a standard curve. Each standard (0.4ml) was dried by rotary evaporation and treated as for the eluates. A blank for the standards consisted solely of 25% acetic acid.

RESULTS

The absolute lipid level found in the gallbladder bile of individual Zambian subjects are shown in Table 1 together with the percentage molar ratio of each of the lipids present. The mean and standard deviation of these values are shown in Table 2 along with results obtained by other workers (Heller and Bouchier, 1973) for Caucasian normal subjects and stone formers. The Zambian specimens contain about half the level of cholesterol and two thirds the level of bile salts normally present in the Caucasian bile but have phospholipid concentration which is greater than the Caucasian level by about a third.

The percentage molar composition of the individual Zambian specimens are shown on the triangular co-ordinate plot (Fig. 1) as solid circles. The areas representing the ranges of biliary lipid composition for Caucasian normal subjects and stone formers are also shown. The composition of three Caucasian gallbladder biles and a Caucasian hepatic bile, measured to provide a direct comparison, are shown on the triangular plot as solid and open triangles respectively. Figure 1 shows that the analytical values found for the four Caucasian specimens agree with the established ranges for Caucasians, and also that the composition of the majority of Zambian specimens lie considerably outside these ranges. The factor most responsible for this large displacement on the triangular plot is the increased phospholipid level.

DISCUSSION

Cholesterol is totally insoluble in aqueous solutions but can be solubilized by a mixture of lecithin and bile salts to form mixed micelles. The capacity of micelles to retain cholesterol in solution is related to the relative amounts of lecithin and bile salt present and the individual effects of these substances can best be demonstrated by expressing all three lipid components (as percentage molar ratios) on triangular co-ordinates (Small, 1968) as shown in Figure 1. All mixtures falling in the area below the curved line crossing the lower part of the graph are less than saturated with cholesterol whereas mixtures falling on or above this line are saturated with cholesterol and will deposit the lipid. The two enclosed areas shown represent the ranges of values established for normal and stone forming Caucasians.

The area occupied by values for Zambians is displaced, relative to that for normal Caucasians, in a direction opposite to the displacement seen for stone former. This result is in line with the observed paucity of cholesterol gallstone disease in Zambia and strengthens the evidence that gallstone disease is related to biliary lipid composition.

The mean biliary cholesterol level in the Zambian subjects was found to be lower than in Caucasians and it could be that this is the important factor in the reduced incidence of gallstones in Zambia. The observation that there is a combined reduction in the levels of cholesterol and bile salt supports the hypothesis that a high intake of dietary fibre decreases intestinal reabsorption of bile salts thereby reducing biliary bile salt levels and secondarily reducing cholesterol levels by the consequent increase in its catabolism to bile salt.

The reduced levels of bile salts in Zambian bile would result in a corresponding reduction in capacity to retain cholesterol in solution were it not for the fact that there is a large increase in biliary lecithin relative to the Caucasian value. This raised phospholipid level increases the cholesterol dissolving power of Zambian bile and is also the factor most responsible for the large displacement in position of these specimens on the triangular co-ordinate plot.

These results raise the question as to which factors play the most important role in preventing gallstone disease, i.e. is it the reduced cholesterol level or the increased phospholipid? Recent studies on the diurnal variation of biliary lipids have shown (Northfield and Hofmann, 1973) that the secretion of lithogenic bile by the liver appears to be a physiologicalphenomenon at the low bile acid fluxes occuring during the physiological interruption of the enterohepatic circulation associated with fasting and sequestration of the bile acid pool in the gallbladder. Whereas cholesterol secretion declines linearly with decreasing bile acid fluxes lower than 11 μ mole/kg/hr the molar ratio of phospholipid to cholesterol actually becomes less than one. The initially higher biliary phospholipid level found in Zambian bile may lessen or prevent the extent to which lithogenic bile acid flux and thereby greatly reduce the incidence of gallstone disease.

TABLE 1: Gallbladder bile in Zambians.

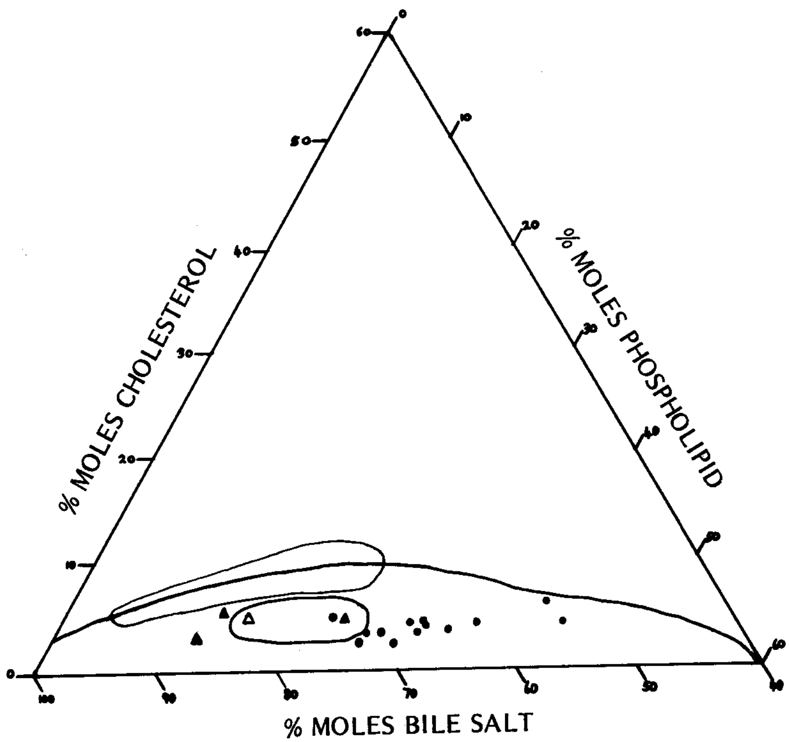
	Absolute Lipid Level (mM/100ml)			Percentage Molar Composition		
	Cholesterol	Phospholipid	Bile Acid	Cholesterol	Phospholipid	Bile Acid
Male subjects						
1.	0.452	4.63	5.88	4.1	42.2	53.7
2.	1.180	9.78	17.30	4.2	34.6	61.2
3.	0.298	3.31	9.15	2.3	26.0	71.8
4.	0.905	8.34	16.30	3.6	32.7	63.8
5.	0.802	3.77	12.10	4.7	22.5	72.2
6.	0.414	3.57	8.98	3.2	27.6	69.4
7.	0.336	4.30	10.30	2.3	28.8	69.0
8.	0.983	6.61	9.15	5.9	39.5	54.6
9.	0.736	6.31	13.70	3.6	30.3	66.1
10.	1.060	7.40	–	–	–	–
Female Subjects						
1.	0.465	3.31	7.18	4.3	30.2	65.6
2.	0.517	3.55	8.16	4.2	29.0	66.6
3.	0.517	3.73	8.00	4.2	30.5	65.4
4.	0.349	2.98	7.84	3.1	26.8	70.3
5.	0.311	3.60	–	–	–	–

TABLE 2: Gallbladder bile in Zambians and Caucasians.

	Absolute Lipid Level (mM/100ml)			Percentage Molar Composition		
Subjects	Cholesterol	Phospholipid	Bile Acid	Cholesterol	Phospholipid	Bile Acid
Zambian	6.2±3.0	49.9±21.1	103.2±35.5	3.8±1.9	30.8±5.4	65.4±5.9
Caucasian	13.0±8.5	33.9±12.3	145.9±56.9	6.9±3.4	17.6±2.7	75.3±5.5
Stone Formers	14.6±7.3	33.3±18.0	137.2±65.4	8.6±3.8	17.6±5.9	73.7±7.7

Figure 1: Plot of percentage molar composition of lipids in bile.

Key: ● = Zambian gallbladder bile
▲ = Caucasian gallbladder bile
△ = Hapatic bile from a stone former taken during cholecystectomy



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