Calcium carbonate in human gallstones and total CO₂ in bile

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SUMMARY Measurement of total CO₂ concentrations in bile from patients undergoing cholecystectomy because of gallstones has shown that the presence of calcium carbonate in the stones can be associated with a raised total CO₂ concentration in the common duct bile. In bile from functioning and poorly-functioning gallbladders, total CO₂ was nearly always related to pH irrespective of stone composition.

The different forms of calcium carbonate (vaterite, aragonite, and calcite) are important constituents of gallstones (Sutor and Wooley, 1971). At University College Hospital, London, we are finding that a higher proportion of patients have gallstones consisting of cholesterol + calcium carbonate than of pure cholesterol, and we want to determine how and why calcium carbonate crystallises from bile. In the first instance, the concentrations of the relevant ions have been measured in bile from different types of stone-formers, to see if they are higher in patients with calcium carbonate in their gallstones. Only this kind of comparison was possible because bile from normal persons was not available. So far, we have found no significant difference in calcium concentrations in bile from calcium carbonate stone-formers and from patients with cholesterol gallstones (Sutor and Wilkie, 1977). In this paper we have investigated some factors influencing the concentration of the carbonate ion.

Over the biliary pH range, which is 6.8-8.05 (Sutor and Wilkie, 1976), the carbonate concentration is approximately 10⁻² to 10⁻³ of the bicarbonate concentration and is, therefore, too small to measure. The bicarbonate concentration can be measured directly by titration but this was not possible for the small amount of bile, particularly common duct bile, collected at operation. We have, therefore, measured the amount of total CO₂ present

which is the sum of the concentrations of bicarbonate, carbonic acid, dissolved CO₂, and carbonate. On searching the literature for bicarbonate concentrations measured by other workers, we have found that they too have measured total CO₂ and called it bicarbonate, or calculated a theoretical bicarbonate value using constants derived for serum. This is not correct as the solubility of CO₂ in bile has not been determined, the pressure of CO₂ with which bile is in equilibrium is not known, and the pK₁ value for the dissociation of carbonic acid in bile is unknown. Until these factors have been investigated, and they could vary from one patient to another, such results should be presented in terms of total CO₂.

Methods

COLLECTION AND SELECTION OF BILE SAMPLES
Bile was collected anaerobically at 37°C from patients undergoing surgery, usually cholecystectomy, at University College Hospital, London, during the period 1974-77. A previously reported technique was used by the surgeons to collect common duct and gallbladder bile and to minimise contamination of the former by the latter (Antsaklis *et al.*, 1975). For the anaerobic collection, bile was drawn into a closed sterile syringe and air from the Luer fitting was immediately expelled. The gallbladder was completely aspirated to minimise stratification effects (Tera, 1960). For technical reasons, common duct bile was not always obtained. The syringe was sealed by bending the needle and insulated to prevent heat loss till it could be placed, generally within 30 minutes, in a constant temperature box at 37°C. In order that bile could be handled relatively

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easily at 37°C, the box used was a dry box fitted with a heating element, fan, and variable transformer. Work on the bile was started immediately.

Eighty-two patients presented for operation, but material suitable for analysis was not always obtained. In the case of gallbladder bile, 11 patients had bile too thick to handle, 13 had no bile in their gallbladders, four had had previous cholecystectomies, and five biles contained blood and were discarded. The remaining 49 samples were analysed.

Forty samples of common duct bile were collected but to obtain data as 'normal' as possible, the following were excluded: bile containing blood, bile from patients with an obstructed or dilated duct, bile obviously containing mucus which suggested it was contaminated with gallbladder bile, and samples less than 200 µl which were unlikely to be anaerobic. These restrictions left 28 samples for analysis. Both common duct and gallbladder bile suitable for analysis were obtained from 15 patients.

pH MEASUREMENTS

The pH was measured using microelectrodes in the constant temperature box and a Radiometer pH meter. The meter was standardised with Radiometer buffer, pH = 7.38 ± 0.02 at 38°C, the reliability of the electrodes being checked regularly. Exposure to air of bile in the glass electrode did not affect the measurement, which was stable for at least 10 minutes. Two measurements were made on each bile. After each determination, the glass electrode was flushed out with saline and water, and the standardisation of the meter was checked. Millipore filtration of bile (described below) did not affect the pH, which could therefore be determined on filtered and unfiltered samples. Most of our pH measurements were made on unfiltered bile.

MILLIPORE FILTRATION OF BILE

To remove any stone debris, precipitate, and soluble and insoluble mucus of high molecular weight, the bile was filtered anaerobically at 37°C through a prefilter and Millipore filter (pore size, 0.45 µm) by attaching to the syringe containing the sample a Millipore stainless steel microsyringe filter-holder with the dead space filled in. All biles analysed were forced through the filters by hand pressure and the filtrate passed into a piece of silicone rubber tubing attached to the outlet on the filter-holder. Samples for analysis were withdrawn through the wall of the tubing using a microsyringe. The first 50 µl would have been in contact with air in the filter-holder and was not used.

MEASUREMENT OF TOTAL CO₂

Total CO₂ was determined at room temperature

with a Technicon Auto-Analyser using a micro CO₂ procedure (Technicon Methodology N-19a). This method is for serum and the following modifications were made for bile:

1. Hydrochloric acid was used instead of sulphuric acid to prevent any calcium sulphate being precipitated, because the calcium concentration in bile is frequently higher than in serum (Sutor and Wilkie, 1977).

2. Water free of CO₂ (made by degassing distilled water) was used as a diluent for the bile instead of saline, as water added to bile does not precipitate proteins or other substances at the dilutions used below.

3. Standardised sodium carbonate solution was added to bile samples having a low pH, which experience showed corresponds to a low total CO₂ concentration, to bring the concentration into the range covered by the standards (1-4 mmol/l).

Samples for measurement were prepared by adding gallbladder bile (100 µl and 200 µl together with sodium carbonate solution if necessary) or common duct bile (100 µl) to a small amount of water free of CO₂ in 2 ml volumetric flasks and making to volume with such water. Measurements were made on unfiltered bile not containing crystals and its Millipore filtrate to confirm that the total CO₂ content was unaffected by this process.

SUBDIVISION OF DATA

The data were considered in relation to two variables.

Gallbladder function

Patients were always divided according to their gallbladder function, which was usually known and had been assessed by oral x-ray cholecystography. Three groups were recognised: patients with functioning, poorly-functioning, and non-functioning gallbladders depending on whether the gallbladder gave good concentration, some concentration, or no concentration of the dye respectively. All patients with an obstructed duct were taken as having non-functioning gallbladders. Data for five patients whose gallbladder function was not determined have not been included and this reduced the number of gallbladder bile samples to 44.

Stone composition

Most patients had gallstones, and the crystalline constituents present were determined by the x-ray powder method (Sutor and Wooley, 1971). Patients were classified according to the surface composition of their gallstones as this area is most likely to be related to the present bile composition. The following grouping was made: (1) pure cholesterol, (2) some

calcium carbonate, usually with cholesterol, (3) calcium salt other than calcium carbonate.

STATISTICAL TESTS

Statistical significance of differences between two groups was determined using the non-parametric Mann-Whitney U-test (Siegel, 1956). To determine if two variables were related, the significance of the correlation coefficient (r) was tested (Pearson and Hartley, 1966). The 5% level was adopted for significance tests.

Results

The estimated errors in the measurements were ± 2 mmol/l for total CO_2 and ± 0.05 for the pH.

Measurements made on biles free of crystals and their filtrates showed that, as expected, the concentration of total CO_2 was not affected by Millipore filtration.

COMMON DUCT BILE

In the case of common duct bile, patients with functioning and poorly-functioning gallbladders were taken together, as the values were unlikely to be affected by the degree of function. Total CO_2 was within the range 22.5-27.7 mmol/l and the mean was 24.5 (SEM, 0.5) for the 10 patients with only cholesterol on the stone surface (Fig. 1). Nine of the gallbladders were functioning and one was poorly-

functioning. Total CO_2 was within the range 29.1-42.5 mmol/l and the mean was 33.9 (SEM, 1.5) for the 11 patients having some calcium carbonate on the stone surface (Fig. 1). Ten of these gallbladders were functioning and one was poorly-functioning. These two groups of stone-formers differ significantly ($P < 0.001$). Only one patient had a total CO_2 concentration in the higher range and no calcium carbonate in the gallstone, which consisted entirely of calcium bilirubinate. This was the only patient from whom common duct bile had been obtained and who had a calcium salt other than calcium carbonate on the stone surface. Total CO_2 for one patient with no gallbladder and one patient with no stone at operation and a functioning gallbladder was 26.6 and 27.6 mmol/l respectively. These values were comparable with those for the cholesterol stone-formers. For the four patients with non-functioning gallbladders, total CO_2 was within the range 17.4-29.4 mmol/l and the gallstone surfaces consisted of cholesterol.

The pH of common duct bile from all patients was within the range 7.5-8.0. There was no statistically significant correlation between pH and total CO_2 for either the cholesterol or calcium carbonate stone-formers with functioning or poorly-functioning gallbladders ($P < 0.16$ and $P < 0.66$ respectively).

GALLBLADDER BILE

Total CO_2 was always less in gallbladder bile than in the corresponding common duct bile irrespective of gallbladder function and stone composition. Table 1 gives values for the 15 pairs of measurements.

Fig. 1 Total CO_2 in common duct bile for patients with functioning or poorly-functioning gallbladders and gallstones of different composition.

Table 1 Total CO_2 concentrations in corresponding common duct and gallbladder bile

Total CO_2 (mmol/l)		Composition group	Gallbladder function
Common duct bile	Gallbladder bile		
24.0	6.5	1	f
25.4	21.3	1	f
24.2	11.1	1	f
25.4	23.2	1	f
27.7	14.0	1	f
23.5	15.4	1	f
22.5	12.9	1	f
30.4	23.6	2	f
40.9	35.6	2	f
31.0	29.2	2	f
42.1	33.2	2	f
38.3	25.4	3	pf
28.5	4.0	1	nf
29.4	14.4	1	nf
27.6	10.8	ns	f

Key to composition of gallstones: 1 = cholesterol, 2 = some calcium carbonate, 3 = calcium salt other than calcium carbonate, ns = no stone. Key to gallbladder function: f = functioning, pf = poorly-functioning, nf = non-functioning.

In Fig. 2, total CO₂ for bile from functioning gallbladders is plotted against pH. Measurements were available only for the cholesterol and calcium carbonate stone-formers. For these patients, the correlation coefficient for total CO₂ regressed on pH was significant ($r = 0.90$, $n = 27$, $P < 0.001$).

Fig. 2 Plot of total CO₂ against pH for bile from functioning gallbladders. The regression line has a slope of 22.2 and intercept of -145.1. The three points lying well above the line were not included in this calculation.

The slope of the line was 22.2 and the intercept -145.1. Three patients with some calcium carbonate on the surface of their stones had values well above the line and these were not included in this calculation. There was also a statistically significant correlation for total CO₂ regressed on pH for bile from the six poorly-functioning gallbladders ($r = 0.84$, $n = 6$, $P < 0.04$). The slope of the regression line was 37.7 and the intercept was -250.4. All of these patients had gallstones with some calcium carbonate, calcium phosphate, or calcium bilirubinate on the surface. There was no statistically significant difference between the regression coefficients corresponding to measurements for patients with functioning and poorly-functioning gallbladders. Data for the latter group are given in Table 2 together with values for patients with non-functioning gallbladders.

Table 2 pH and total CO₂ concentrations in gallbladder bile from patients with poorly-functioning or non-functioning gallbladders

pH	Total CO ₂ (mmol/l)	Stone composition group and comments
Poorly-functioning gallbladders		
7.10	14.7	2
7.15	20.7	3
7.30	25.4	3
7.35	27.6	2
7.40	35.4	2
7.53	29.2	2
Non-functioning gallbladders		
6.90	2.6	3 (blocked duct)
—	4.0	1 (watery bile)
6.65	5.0	1
6.35	8.4	1 (white bile)
6.80	14.4	1
7.48	17.9	1
7.85	18.3	2
7.35	19.2	2

Composition of surface of stones: 1 = cholesterol, 2 = some calcium carbonate, 3 = calcium salt other than calcium carbonate.

Discussion

Although data are limited because of the problems associated with collecting bile from human subjects, some highly significant results have been obtained. For patients with functioning or poorly-functioning gallbladders, the presence of calcium carbonate in gallstones was related to a raised concentration of total CO₂ in common duct bile. Values were within the range 22–28 mmol/l for cholesterol stone-formers and they are within the normal range for serum (20–28 mmol/l, University College Hospital values). It is suggested they represent normal concentrations for common duct bile. Values greater than 29 mmol/l were almost invariably associated with the presence of calcium carbonate on the stone surface and are referred to as raised concentrations. Only one patient had a raised value for total CO₂ and no calcium carbonate in the gallstone which was composed of calcium bilirubinate. This would suggest that, in solutions supersaturated with calcium bilirubinate and calcium carbonate, the former is deposited.

The reason for the raised total CO₂ values in these biles is not known, but it could result from a disturbance in the secretion of secretin. In a study of the rate of bile flow and the electrolyte composition of human hepatic bile in cholecystectomised patients with T-tubes in the common duct, the administration of secretin augmented the flow of common duct bile with a fluid rich in total CO₂ (Waitman *et al.*, 1969). These workers reported that the fluid was rich in bicarbonate, but they, too, measured total CO₂.

In patients with non-functioning gallbladders, two of the total CO₂ concentrations in common duct bile (17.4 and 20.4 mmol/l) were below the values

found for patients with functioning or poorly-functioning gallbladders. This may indicate that the suggested normal range is too small, or that some of the molecules or ions responsible for the total CO_2 concentration have been lost by diffusion through the duct walls. One patient in this group had a total CO_2 concentration of 29.4 mmol/l and this did not lead to the precipitation of calcium carbonate. However, if an increase in the concentration of total CO_2 occurred *after* the gallbladder became non-functioning, and if this bile could not enter the gallbladder, the gallstone composition would not reflect this abnormality in the composition of the common duct bile.

In the gallbladder, there was a decrease in the total CO_2 concentration and this is consistent with the active transport of bicarbonate ions from the gallbladder. However, passive diffusion through the gallbladder wall of molecules and ions, such as dissolved CO_2 and bicarbonate, may also be occurring during concentration of bile in the gallbladder.

Although there was no correlation between total CO_2 and pH in common duct bile, these two variables were related in bile from functioning and from poorly-functioning gallbladders, irrespective of the composition of the gallstones formed. Three of the calcium carbonate stone-formers had values lying above the regression line for data from patients with functioning gallbladders. These high concentrations could have resulted from the gallbladder having emptied not long before the samples were taken, or the gallbladder function may have deteriorated between the time of the cholecystogram and the time of the operation.

Our studies suggest that an important factor in the deposition of calcium carbonate in gallstones is the raised level of total CO_2 in common duct bile. Precipitation would occur when such bile enters the gallbladder before the relationship between pH and total CO_2 has been attained.

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