Presence or absence of inhibitors of crystal growth in bile

1. Effect of bile on the formation of calcium phosphate, a constituent of gallstones

D. JUNE SUTOR AND JOSEPHINE M. PERCIVAL

From the Department of Chemistry, University College, Gower St., London

SUMMARY When calcium and phosphate ions were mixed so that their final concentration was 4 mmol/l and the pH was kept at 7.0, an amorphous precipitate immediately formed and this changed into crystalline material with an apatite-like structure after a period of time. The formation of either or both types of precipitate could be slowed down or prevented by adding to the crystallising medium trace amounts of pyrophosphate or citrate which are known inhibitors of the formation of calcium phosphate, or large quantities of sodium chloride which increased the ionic strength of the solution and hence the solubility of calcium phosphate. Both common duct and gallbladder bile from patients with gallstones composed of cholesterol and/or calcium carbonate had a very pronounced inhibitory action on the formation of these precipitates. Only very small amounts of bile were necessary to produce these effects, which therefore were not due to an increase in ionic strength. Ultrafiltration of bile showed that material with a molecular weight greater than 10 000 was mainly responsible for this activity. Because the inhibitor was present in both common duct and gallbladder bile, the liver is the likely source of origin. The possible identity of this material is examined. The powerful inhibitory effect of bile on the crystallisation of calcium phosphate is probably a contributory factor to the rare occurrence of the calcium phosphates, apatite and whitlockite, in gallstones.

Sutor and Wooley (1973) have recently shown that calcium salts are often important constituents of gallstones. In their study of the crystalline composition of 481 stones from 11 countries, only 59.9% of the gallstones consisted entirely of cholesterol, 27.0% contained both cholesterol and calcium salts in varying proportions, and 13.1% were composed of calcium salts. The calcium salts found were vaterite, aragonite, and calcite (different crystalline forms of calcium carbonate), apatite and whitlockite (calcium phosphates), calcium palmitate, and calcium bilirubinate. Conditions leading to or preventing their nucleation and crystal growth from bile have not been investigated, and we have therefore initiated a programme of research designed to study these factors.

It has long been known that the addition of certain compounds or ions to a crystallising medium can have a profound effect on the growth rate of the

crystals and on their composition. Those substances which slow down or prevent crystal growth are commonly referred to as inhibitors, and they can act in several ways. Any compound or ion which forms a suitable stable complex with any of the ions which would normally precipitate out, will reduce the rate of crystallisation or even prevent crystallisation occurring depending on the amount present. The requirements which must be fulfilled by a suitable complex are that it is soluble and that its stability constant is greater than that of the salt which would normally precipitate. Prevention and retardation of crystal growth may also be accomplished by the addition of compounds and ions which are adsorbed on to certain crystal faces and so cause habit modification and an alteration in the rate of crystal growth.

The presence or absence in bile of such an inhibitor might explain why, when other things are equal, a particular calcium salt is formed by one person and not by another. It is therefore important

that bile should be tested for the presence of such substances, and this paper reports our studies on the inhibitory action of bile on the nucleation and crystal growth of calcium phosphate. Apatite and apatite-like compounds which turn into apatite on standing were the calcium phosphates chosen since whitlockite is difficult to prepare in the laboratory.

Method

The basis of the technique used to test for the presence or absence in bile of substances which affect the crystallisation of calcium phosphate is the well-known fact that precipitation of apatite from aqueous solutions proceeds through an amorphous to a crystalline phase and the formation of the amorphous and crystalline material is accompanied by a fall in pH (see references in Francis, 1969; Elliott, 1973).

The experimental method adopted was as follows. Ten millilitres of a solution of disodium hydrogen phosphate (0.02 mol/l) were mixed with 30 ml water and the pH adjusted to 7.0 with normal hydrochloric acid. This pH value was chosen as it is a roughly average value for bile. Ten millilitres of a solution of calcium chloride dihydrate (0.02 mol/l) were then added to the solution, which was stirred magnetically and which contained the electrodes for monitoring the pH. The pH of the solution was kept within the range 7.0 ± 0.1 by adding, when necessary, normal sodium hydroxide from a microsyringe, and the amount added was plotted against time. Analar reagents and deionized water were used throughout.

Precipitates were collected at different times during the reaction, and, to arrest the crystallisation process, they were obtained by rapid filtration under pressure, washed quickly with water, and dried with acetone. The composition of the material and whether it was amorphous, crystalline, or a mixture of both states were determined by X-ray diffraction (Sutor and Wooley, 1971).

The effect of inhibitors in this system was examined by adding varying amounts of sodium citrate or sodium pyrophosphate to the water so that the final volume did not exceed 30 ml. Citrate forms a soluble complex with calcium, and pyrophosphate is adsorbed on to crystal faces preventing further growth on them. The effect of ionic strength on the solubility of calcium phosphate was tested by adding sodium chloride.

Samples of bile from gallbladders and common bile ducts were collected anaerobically in syringes during cholecystectomies. The bile was immediately filtered anaerobically at 37°C using a stainless steel filter holder (Millipore Ltd.) containing a filter (pore size, 0.45 μ m) and prefilter. This removed any mucus

or crystalline material. The bile was introduced as soon as possible, generally within the hour, into the crystal growth system. As in the above experiments, it was added to the water, and control runs were determined for every sample tested.

Filtered bile was ultrafiltered anaerobically at 37°C through a PM-10 filter (Amicon Ltd.) which has a nominal molecular weight cut-off of 10 000. Details of our specially-built apparatus to ultrafilter bile anaerobically will be published elsewhere. Bile and its ultrafiltrate were also tested in the crystal growth system.

Sodium taurocholate (Calbiochem) and egg lecithin were shaken in water to give a micellar solution in which their relative concentrations were 100 and 30 mmol/l. Aliquots of this solution were tested in the crystal growth system.

Experiments were carried out on inhibitors and bile both at 37°C and at room temperature (21–23°C). The increase in temperature increased the rate of reaction and the amount of material required to inhibit the reaction but the net overall effect was the same in all cases. Room temperature was therefore chosen because it was more convenient.

Results

When calcium phosphate was precipitated in this system in which the initial concentrations of calcium and phosphate were 4 mmol/l and the pH was 7.0, the plot of time against added sodium hydroxide is shown in the Figure. Amorphous flocculant material was produced immediately as the first drop in pH took place, but within a few minutes some ordering occurred in some of the precipitate and a little crystalline material was formed. During this period the precipitate still consisted of flocculant particles, but these became smaller, denser, and crystalline in appearance at the second change in pH. When the pH no longer needed adjusting, the precipitate consisted of crystalline material which had a diffraction pattern closely similar to that of apatite. However, the *d*-spacing of one of the lines was slightly different from the corresponding one in apatite and hence this substance has been referred to in this paper as apatite-like material. On standing, it could be converted to apatite or octacalcium phosphate depending on the pH of the medium in which it was kept.

The addition of sodium citrate, pyrophosphate, or chloride to the test system could alter this pattern of events. Only small amounts of citrate and pyrophosphate of the order of 10^{-5} mol/l were necessary to delay the formation of the crystalline phase for about 20 minutes (Table 1). (For comparing different test runs, the time corresponding to the mid-point of the curve representing the second change in pH was

Figure The effect of 80 μ l bile and its ultrafiltrate on the formation of calcium phosphate followed by plotting the amount of sodium hydroxide required to keep the pH at 7.0 when calcium and phosphate ions are mixed so that their final concentrations are 4 mmol/l.

found to be the most convenient). On the other hand, an added concentration equivalent to 1.8×10^{-2} mol/l of sodium chloride was necessary for the ionic strength to affect the reaction (Table 1).

The presence of bile in the test system also had a very great effect on the formation of the apatite-like material (Figure, Table 2). Both gallbladder and common duct bile inhibited the formation of the crystalline material. In one instance, a concentration of 0.16% gallbladder bile in the test delayed crystallisation for 24 minutes and inhibited the formation of the amorphous phase for 18 minutes. Larger quantities of some gallbladder biles oc-

casionally prevented the formation of the initial amorphous precipitate, and in one case the solution remained clear for 24 hours, after which it was discarded because of possible changes in the bile caused by enzymes and bacteria.

Table 3 and the Figure show the results when gallbladder bile and its ultrafiltrate were introduced into the test system. All the biles caused considerable inhibition in the formation of the crystalline precipitate, and two biles delayed the appearance of the amorphous material (in one instance no precipitate formed during the experiment). However, an equivalent volume of ultrafiltrate usually showed very

Table 1 Effect of sodium chloride, pyrophosphate, and citrate on crystallisation of apatite-like calcium phosphate

<i>Final concentration of added material (mmol/l)</i>	<i>Time (min) to form</i>	
	<i>Amorphous precipitate</i>	<i>Crystalline precipitate</i>
a. Sodium chloride		
—	0 ¹	19
15	0	21
18	0	27
90	17	No CP after 60 min when run stopped
b. Sodium pyrophosphate		
—	0	19
0.01	0	40
0.02	0	{ No CP after 60 min when run stopped
0.2	0	
c. Sodium citrate		
—	0	19
0.035	0	35
0.7	5	80

¹0 indicates instant precipitation.
CP: crystalline precipitate.

Table 2 Effect of bile on formation of apatite-like calcium phosphate

Amount of bile in test (ml)	Time (min) to form			
	Amorphous precipitate in		Crystalline precipitate in	
	Water	Bile	Water	Bile
a. Gallbladder bile				
1	0	NP after 90 min	16	—
0.13	0	12	18	38
0.75	0	0	15	100
0.7	0	NP after 24 h	15	—
1	0	0	16	19
2.5	0	0	16	39
0.08	0	18	16	40
b. Common duct bile				
1	0	0	18	No change after several hours
1	0	0	18	No change after 3 h

NP: no precipitate.

Table 3 Effect of bile and its ultrafiltrate on formation of apatite-like calcium phosphate

Amount of bile or its ultrafiltrate in test (ml)	Time (min) to form					
	Amorphous precipitate in			Crystalline precipitate in		
	Water	Bile	Ultrafiltrate	Water	Bile	Ultrafiltrate
0.75	0	NP after 24 hr	3	16	—	23
0.08	0	18	0	16	40	20
0.5	0	0	0	18	65	48

little inhibitory power. In two cases the behaviour of the ultrafiltrate was almost comparable with the water control but in the other the ultrafiltrate retained some inhibitory power.

A concentration of 1 mmol/l sodium taurocholate and 0.3 mmol/l lecithin in the test system delayed the formation of the crystalline phase for 90 minutes, at which stage the experiment was stopped and concentrations of these substances of 0.6 and 0.2 mmol/l respectively inhibited this change for 60 minutes.

Discussion

Both gallbladder and common duct bile inhibited the formation of calcium phosphate in our test system. The 12 different biles tested all had this effect but some showed greater inhibitory power than others. Calculations based on average concentrations of electrolytes in bile showed that the amount of bile used had a negligible effect on the ionic strength of the crystallising medium. This was confirmed by ultrafiltration. The monovalent and divalent ions which are largely responsible for the ionic strength pass through the ultrafilter but the ultrafiltrate usually showed little inhibitory power. Very little is known about the concentration of calcium and phosphate ions in bile. However, it readily follows from the small amount of bile used in the test system, that the effect did not result from simple unsaturation of the bile with respect to calcium phosphate. The common duct and gallbladder bile tested therefore contained a substance or substances which behaved like citrate and/or pyrophosphate in the test system. Ultrafiltration showed that the material was a large molecule with a molecular weight usually greater than 10 000. A variation in molecular weight or a degradation product which passed through the ultrafilter could explain the inhibitory effect shown by one ultrafiltrate.

Because the inhibitor was present in both common duct and gallbladder bile, the liver is probably its source of origin. The isolation of this material is likely to be difficult as bile is unstable, and we have therefore attempted to determine its identity indirectly. The high molecular weight constituents of bile most likely responsible for the observed effect are the

bile salt/phospholipid micelles. Such polyvalent anions particularly those containing sulphate or phosphate groups have a strong affinity for divalent and trivalent cations and would therefore bind calcium. Moreover, polyvalent anions can frequently inhibit crystal growth through adsorption on to crystal faces like the well-known inhibitors heparin and sodium polymethacrylate. Indeed, in our test system a pronounced inhibitory effect on the formation of calcium phosphate was shown by small amounts of a micellar solution of sodium taurocholate and lecithin in which the bile salt and phospholipid concentrations were comparable with those in an average bile.

It is unlikely that any of the other known biliary constituents of high molecular weight would inhibit the formation of calcium phosphate apart from albumin, the calcium-binding protein of blood, and the small amount present could not account for the effect observed. However, only a correlation of the inhibitory power of bile with that of bile salt/phospholipid solutions of comparable composition or the isolation of the inhibitor from bile will show if the micelles are the only compounds involved.

All biles used in these experiments came from patients with gallstones composed of cholesterol and/or calcium carbonate, the majority of stones containing both these compounds. So far, bile from a patient with calcium phosphate stones has not been available for testing in our system. As mentioned earlier in this paper, apatite and whitlockite are rare constituents of gallstones. The strong inhibitory action of bile on the crystallisation of the apatite-like material is likely to be an important contributory factor to the infrequent occurrence of calcium phosphate in gallstones.

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