A structural study of gallstones

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SUMMARY   A number of gallstones have been studied using methods which have not previously been applied to gallstones. In particular, the use of scanning electron microscopy and micro-*x*-radiography have allowed detailed observations to be made on the structure of the stones and the distribution of the various components within the stones. Large differences in structure have been shown to exist between stones having similar overall chemical compositions. In cholesterol gallstones containing calcium carbonate the crystalline nature, distribution and method of deposition of the calcium carbonate was studied and was found to vary from stone to stone. Evidence was found for the presence of fibrous material in the centre of many stones and it is possible that this material acted as a nucleus for the deposition of the other stone components.

Previous studies carried out on the chemical composition of gallstones by Sutor and Wooley (1971) and by Rains, Barson, Crawford and Shrewsbury (1960) have shown that most human gallstones consist mainly of cholesterol. These cholesterol stones often contain small quantities of other substances such as calcium carbonate and pigment material. Much less common is a second category of gallstones consisting almost entirely of calcium carbonate. A third type of stone contains the calcium salt of the bile pigment, bilirubin, and a fourth type consists of organic material other than cholesterol. Despite these surveys on the composition of gallstones, very little has been published on the structures of individual stones. It was therefore decided to conduct a detailed study of a small representative number of gallstones using a range of techniques including scanning electron microscopy, optical spectroscopy, micro-*x*-radiography, chemical analysis and *x*-ray diffraction. It was intended to obtain information relating to the nucleation of the stones and the growth of the layered structures.

Materials and Methods

Gallstones were obtained from St Luke's Hospital and the Royal Surrey Hospital at Guildford. No details of the patients or of their case histories were made available. A large number of stones were examined, but the stones from 10 patients were examined in detail and their main characteristics are summarized in the table. The number of stones

per patient varied from 1 to 80. Most of the stones consisted mainly of cholesterol, and all contained pigment material, especially at the centre. Patients A-G had cholesterol stones, H had a calcium carbonate stone, J had a calcium bilirubinate stone and K had an organic stone.

IDENTIFICATION OF CONSTITUENTS

To determine the amount of cholesterol present a weighed stone, or known fraction of a stone, was treated with benzene to dissolve out the cholesterol. The residue was filtered, washed, dried and weighed. The benzene solution was evaporated and the cholesterol which remained was weighed and identified by *x*-ray diffraction and melting point. The part of the stone which was insoluble in benzene was examined by *x*-ray diffraction, when crystalline components such as calcium carbonate, calcium hydrogen phosphate, apatite and sodium chloride were identified. Subsequent treatment of this residue with dilute hydrochloric acid dissolved out the inorganic components and left the amorphous pigmented organic material. When applicable, infrared analysis was used to identify calcium bilirubinate or to confirm the absence of calcium bilirubinate.

In many stones an outside layer, 0·3-1·0 mm thick, had a different appearance from the rest of the stone and could easily be detached and examined separately. Similarly, a central pigmented area could often be distinguished from the rest of the stone. This was removed and analysed separately.

SCANNING ELECTRON MICROSCOPY

The use of the scanning electron microscope allowed

Patient	Approximate Size of Stones (cm)	No. of Stones	Approximate Percentage of Cholesterol	Calcium Carbonate Present			Structure
				Calcite	Aragonite	Vaterite	
A	1·2	1 Solitaire	90	Some	Mainly	Little	CaCO₃ shell. Layered interior like a tree trunk
B	1·3	1 Solitaire	96	Little	0	Mainly	Large cholesterol crystals lying along stone radii
C	0·2–1·0	31	98	Some	Some	Mainly	Heavily pigmented centre with no layer structure
D	0·2–0·6	70	99	Little	0	0	Heavily pigmented centre with no layer structure
E	0·8–1·5	4	95	Little	Little	Mainly	Radial cholesterol crystals, columns of CaCO₃
F	0·2–0·3	20	>99	0	0	0	Radial cholesterol crystals, heavily pigmented centre
G	0·2–1·0	80	99	Trace	Trace	0	Heavily pigmented centre
H	1·0	1	0	Mainly	Little	Little	Black CaCO₃ stone
J	4·0 × 0·8	1	0	0	0	0	Black calcium bilirubinate stone
K	0·6	20	0	0	0	0	Black organic stone, melting point >320°C

Table *Composition of gallstones*

the physical structure of different parts of the stone to be examined at high magnifications (up to × 50 000). Pieces of gallstone, or fragments of stone, from which the cholesterol had been removed by treatment with benzene, were coated in a vacuum coating unit with a thin conducting layer of gold-palladium alloy and then examined in a Cambridge mark IIa scanning electron microscope. The micrographs were recorded on 35 mm film. Any part of the stone—outside surface, fracture surface, centre, radius, etc—could thus be examined since the method proved to be very flexible.

MICRO-X-RADIOGRAPHY
Thin slices of stone were prepared from suitable stones by cutting sections with a scalpel and then rubbing the slices on fine emery paper, or on flat alumina surfaces, until thin enough for microradiography (0·2–0·5 mm). Some stones were too friable to be treated in this manner, so that micro-x-radiographs could not be obtained for all stones. The thin sections were backed with a piece of high resolution photographic plate and placed in a small light-tight box having an aperture for the incident x-ray beam. Copper radiation was used at 10 kV and 5 ma from a crystallographic x-ray set, and the specimen was irradiated for about two to five minutes depending on the thickness and composition of the sample. The microradiographs obtained could be enlarged to show details of the denser parts of the stones, thus giving, for example, the distribution of dense calcium carbonate in cholesterol stones and the distribution of voids in a calcium carbonate stone. These radiographs, when used in conjunction with the other techniques, such as x-ray diffraction and

scanning electron microscopy, helped to build up a more complete picture of the overall stone structure.

POLISHED SECTIONS OF STONES
It was found useful in many cases to study the distribution of pigment in a stone by cutting the stone and polishing the broken face to a smooth surface on fine emery and finally on filter paper. When viewed under an optical microscope at a magnification of × 40, any layer structure in the stone was visible, and the width of each band could be measured and the distribution of pigment observed. The observations were recorded on 35 mm colour film using a camera attached to the microscope. This film could then be enlarged further if required. This polishing procedure was found to be essential to reveal the existence and the details of the layer structure, which were not always obvious on just breaking the stones open.

Results and Discussion

The results are given and discussed for each type of stone studied.

SINGLE CHOLESTEROL STONES
Patients A and B each had one large cholesterol solitaire stone. These stones were roughly the same size and shape, but A had a smooth, dark grey-green surface and B had a knobbly, cream-coloured surface. The outer, dark-coloured layer of A was 0·5 mm thick, was easily detached from the rest of the stone and was found to consist mostly of calcium carbonate, whilst the outer layer of stone B was composed only of cholesterol. The internal struc-

tures of the two stones also differed. Stone A had a structure similar to that of a tree trunk—built up of many layers of cholesterol containing patches of pigmented material. The thickness of each layer was 0·01-0·02 mm, and such a layer corresponded to approximately 5 mg of cholesterol. The total amount of cholesterol produced in the bile daily is of the order of 5 g (Hargreaves, 1968) so that the amount deposited in one layer represents about one thousandth of this. The pigment was present as spots in each layer and some layers were more heavily pigmented than others. The centre of stone A was ill defined and seemed to consist of three adjacent heavily pigmented areas. Stone B, on the other hand, had a well defined pigmented centre with large cholesterol crystals radiating out from the centre. The shiny cleavage faces of the crystals could easily be seen in the broken stone. It was readily shown by x-ray diffraction that the cholesterol had grown with an important crystallographic axis (b = 33·7 Å) of the crystal along the radius of the stone.

The detection, by x-ray diffraction, of components other than cholesterol was carried out on pieces of stone from which cholesterol had been removed by treatment with benzene. When the cholesterol was dissolved away, the residue (3·4%) of stone B consisted of small clusters of reddish brown, irregularly shaped crystals and a few colourless crystals. Analysis of this residue by x-ray diffraction showed that it consisted mainly of vaterite and a little calcite. To identify the parts of the stone associated with the small amount of calcium carbonate present, a thin slice of the stone was placed on a microscope slide and a drop of warm dilute hydrochloric acid was added. The effervescence associated with the dissolution of the carbonate was seen to occur where there were dark patches of crystals. Thus, the calcium carbonate seemed to be associated with the pigmented areas of the stone. It was also noted that pure calcium carbonate stones were invariably black or dark brown. A micro-x-radiograph of a thin section of the stone (fig 1a) showed how the denser calcium carbonate was unevenly distributed throughout the cholesterol, most of it being concentrated in one ring. A thin section of another cholesterol-calcium carbonate stone (fig 1b) also showed an uneven calcium carbonate distribution, indicating that conditions in the gallbladder varied considerably during the period of stone growth, as suggested by Sutor and Wooley (1974). The calcium carbonate in stone A was
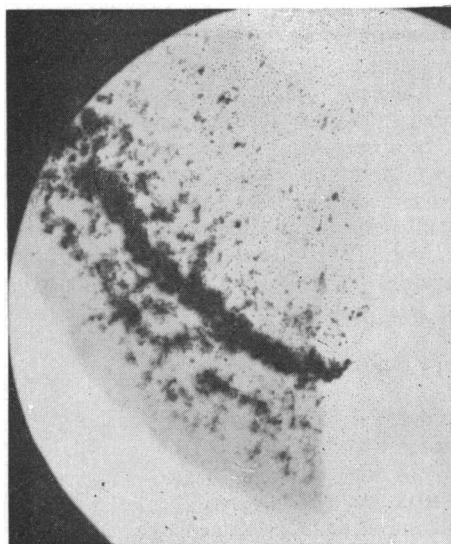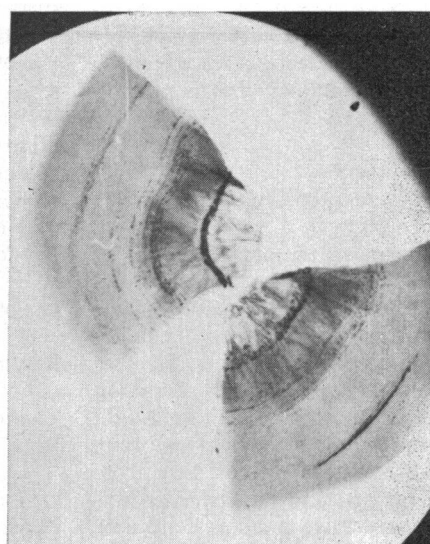

Fig 1a   Fig 1b

Fig 1   *Micro-x-radiographs of thin sections of segments of gallstones*

Fig 1a   *Solitaire B stone × 13*

Fig 1b   *Cholesterol stone containing calcium carbonate × 13*
  Operating conditions, Cu radiation, 10 Kv, 5 ma, two minutes' exposure.
  The non-uniform distribution of calcium carbonate within the cholesterol is clearly shown as dark bands.
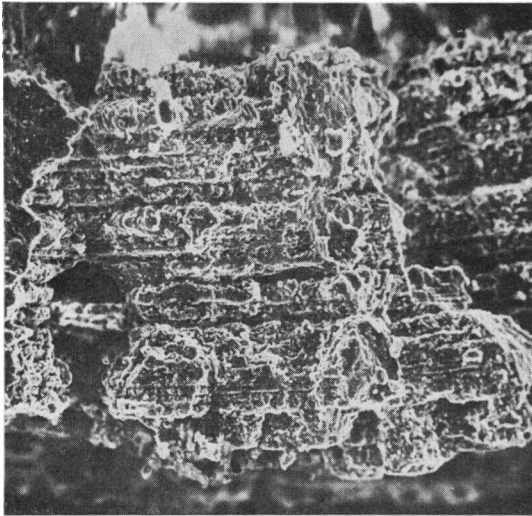
Fig 2 *Scanning electron micrograph of stone A. Fragment of stone, after removal of cholesterol, showing layers of calcium carbonate (largely aragonite).* × 165

shown by micro-x-radiography to be more evenly distributed in many layers about the centre. Figure 2 shows a scanning electron micrograph of the layers of calcium carbonate after the cholesterol had been dissolved out. The fairly evenly spaced layers of calcium carbonate were clearly visible. As can be seen from the table, the calcium carbonate in the solitaire A stone was mainly aragonite with some calcite and only a little vaterite, whereas solitaire B contained mostly vaterite but no aragonite.

The differences in the forms of calcium carbonate present in the two solitaire cholesterol stones have to be considered in terms of what is known about calcium carbonate from work in other fields. Vaterite is an uncommon form of calcium carbonate. The different forms of calcium carbonate may be attributed to different causes. Calcite is known to form at relatively low super-saturation levels and aragonite at higher levels (Kirov and Filizova, 1970). The more unusual vaterite structure is known to be stabilized by impurities such as $BaCO_3$ (Hashizume and Takashima, 1968). Vaterite precipitates at a lower temperature than either calcite or aragonite and the precipitated vaterite is converted to calcite or aragonite if left to age in an aqueous medium (Wray and Daniels, 1957). This implies that those gallstones containing vaterite became quickly covered in cholesterol and thereby isolated from the bile solution so that no recrystallization could take place.

Calcium carbonate may also be formed by bacterial action (Boquet, Boronat, and Ramos-Cormenzana,

1973) and bacteria have been shown to be present in many gallstones (Rains *et al*, 1960). In the present work, calcium carbonate has always been found to be associated with pigmented areas of the stone. This coprecipitation of calcium carbonate and pigmented material may be brought about by a change in the pH of the bile. The pH of the bile is largely controlled by the re-absorption of water, bicarbonate and other solutes by the walls of the gallbladder, and the normal range in pH is from 6·1 to 8·6 (Hargreaves, 1968). If the re-absorption mechanism were not functioning correctly the pH might easily rise to a value of 9, which would probably be high enough to precipitate $CaCO_3$ (Wray and Daniels, 1957), although it is difficult to define the exact conditions necessary for precipitation in such a complex solution as bile. Certainly, calcium carbonate precipitation does sometimes occur in bile to give the condition known as limey bile, but such precipitation does not necessarily lead to stone formation.

The structure of stone A, with its different layers which have been built up gradually, would be the expected structure for a gallstone which started from a small nucleus and grew due to the deposition of cholesterol and small amounts of calcium carbonate. Under these circumstances, aragonite and calcite would be the expected forms of calcium carbonate. The different structure of stone B, with its large crystals of cholesterol radiating from the centre, its calcium carbonate largely in the form of vaterite, and only slight evidence of a layer structure, must arise in a different way. Possibly here all the cholesterol and calcium carbonate might be suddenly deposited from a supersaturated bile solution onto a nucleus instead of building up slowly layer by layer, over a long period of time.

MULTIPLE CHOLESTEROL STONE DEPOSITS
In addition to the cholesterol solitaire stones, multiple deposits of cholesterol stones are common. Examples of such stones are the C, D, E, F and G stones listed in the table. The F stones were very small and spherical with heavily pigmented centres and crystals of cholesterol radiating from the stone centre, and were very similar to the solitaire B stone except that they were much smaller, more numerous and more heavily pigmented. They probably formed in a similar way to the B stone, but nucleated on 20 sites instead of one. Scanning electron micrographs of the pigmented centre of an F stone show a disorientated mass of small flakes and particles of cholesterol and pigmented material, whilst micrographs of the outer parts of the stone show large orientated flakes of cholesterol. Calcium carbonate could not be detected in the F stone, but there was a trace of sodium chloride.
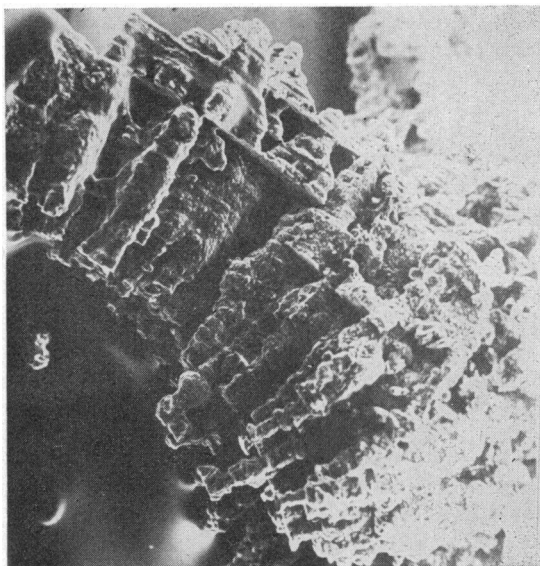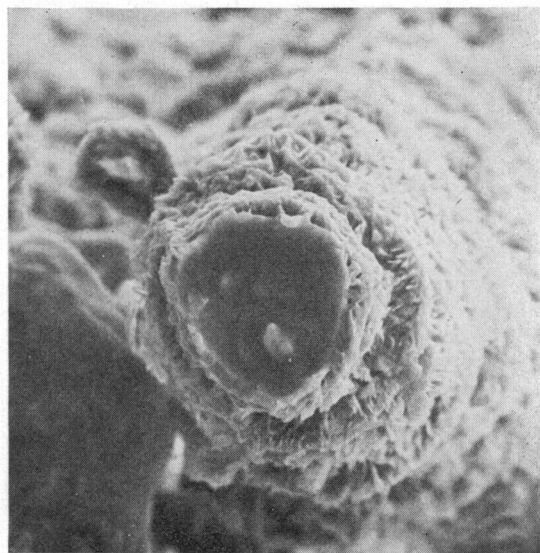
Fig 3a   Fig 3b

Fig 3   *Scanning electron micrographs of gallstone E after removal of cholesterol.*

Fig 3a   *Columns of calcium carbonate (mainly vaterite) from periphery of stone. Layer structure also visible.* $\times$ *45*

Fig 3b   *Top of one of columns in fig 3a, showing small vaterite crystals in layer formation.* $\times$ *660*

The E stones were also structurally very similar to the cholesterol B solitaire stone, and again the calcium carbonate was present almost entirely as vaterite. Furthermore, micro-x-radiographs of thin sections of gallstone E showed that the calcium carbonate was distributed in discontinuous rings around the centre. When the stone was treated with benzene to remove the cholesterol, the structure of the calcium carbonate deposits could be seen as columns radiating towards the centre (fig 3a). However, layer formation was also still visible, and the layers of deposit again seemed to be about 0·01 and 0·02 mm wide. It was shown by x-ray diffraction that the columns of calcium carbonate crystals had not grown with any preferred orientation. Figure 3b shows the end of one of the vaterite columns where the characteristically small vaterite crystals may be seen.

It may be observed that the structure of these vaterite columns is very different from that of the layers of calcium carbonate found in gallstone A (fig 2). This difference in structure, together with the fact that in one case the calcium carbonate is largely aragonite and in the other is mostly vaterite, would reinforce the view that the stones grew by different mechanisms, as indicated by the different macroscopic appearances of the interiors of the stones.

Scanning electron microscopy of a fragment of the centre of the E stone after the cholesterol had been dissolved revealed the presence of spheres and flakes in the mixture of calcium carbonate and pigmented material. No evidence was found for a central small nucleus. The flaky material was the pigment and the spheres were the calcium carbonate.

An examination of the G stones by scanning electron microscope again showed the presence of large flakes of cholesterol away from the centre of the stone. The pigmented centre showed little sign of any systematic structural formation. Figure 4 shows the pigmented residue of the centre after the cholesterol had been dissolved out. Fibrous material is clearly visible in the centre of the stone. The C and D stones also contained fibrous material at their centres. The pigmented centres of these stones were quite distinct areas from the thin, outer, whitish layers, and the two differently coloured parts separated readily. The optical microscope revealed that the centre contained reddish brown crystals and patches of black crystals and no small growth nucleus was visible. The dark central area had no layer structure and contained voids which could have formed when mucus-type material dehydrated and formed fibres. The white outer part of the stones was laid down in layers about 0·01-0·02 mm thick, and some of these layers contained more patches of pigment than others.
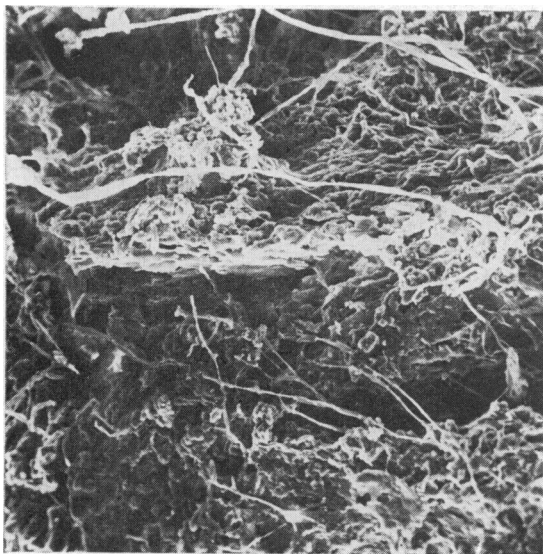
Fig 4 *Scanning electron micrograph of stone G. Centre of stone after removal of cholesterol. Random arrangement of fibres and flaky pigmented material.* × 375

CALCIUM CARBONATE STONE

One calcium carbonate stone was examined (H). It was a large, hard, heavily pigmented stone containing mostly calcite but also some vaterite and aragonite A little brushite ($CaHPO_4.2H_2O$) was also present. No cholesterol was found in the stone. When viewed under the optical microscope the stone was seen to have a porous structure, and the crushed stone was seen to consist of yellowish crystals and a few darker reddish brown crystals.

It may be inferred that a stone which consists almost entirely of pigmented calcium carbonate must be the result of a different physiological disorder from that which produced a stone which consists mostly of cholesterol. Also, the calcium carbonate is accompanied by pigmented material. Throughout the investigation the crystals of calcium carbonate have always been coloured. Normal bile contains calcium ions and bicarbonate ions, but precipitation of calcium carbonate could be brought about by a change in the pH of the bile, which is normally controlled by the re-absorption of the bicarbonate ions and water through the walls of the gallbladder. Any factor which upsets this bicarbonate re-absorption could result in the formation of solid calcium carbonate. The precipitation of calcium carbonate does not, however, explain the formation of a stone. It would be expected that the precipitate would merely form a milky suspension, which would be expelled from the gallbladder with

the bile in the normal way. If, however, the precipitates were trapped in some biliary debris or muco-substances, then stone formation might be able to take place as outlined by Bouchier (1971). Alternatively, if calcium carbonate precipitation occurred over a long period of time, then the stone might be able to grow, despite the filling and emptying of the gallbladder, in the same way that calcium carbonate deposits occur in a kettle.

CALCIUM BILIRUBINATE STONE

The J stone (table) was a long, black, irregularly shaped stone, whose infrared spectrum indicated that it was a calcium bilirubinate stone. Electron probe analysis showed that, in addition to calcium, the stone contained a little phosphorus, sulphur and iron. When the interior of the stone was examined using the scanning electron microscope it was found to consist of flakes of pigment material, but no obviously crystalline shapes were seen and no ordered structure was visible. Bilirubin is normally present in the bile as bilirubin diglucuronide. If, for some reason, this compound breaks down to give bilirubin and a glycoside residue, then the free bilirubin will always react with the calcium ions present to form a precipitate of calcium bilirubinate. It is believed that bacterial infection can be responsible for such bilirubin diglucuronide breakdown (Bouchier, 1971) and hence for some calcium bilirubinate stones.

ORGANIC STONES

The table gives details of one sample of small, shiny black stones, K. They contained no cholesterol or calcium carbonate, but traces of NaCl were found. Chemical analysis showed that they contained C, N and H in the approximate atomic ratio of 12:2:17 (47% C, 9·4% N and 5·4% H). They also contained sulphur but no iron, and were soluble in sodium hydroxide. When heated to 700° in air, they burnt, leaving only a small black residue. The stones were composed largely of organic material, possibly a derivative of an amino acid, such as taurine $NH_2(CH_2)_2SO_3H$ and a pigment material. It was shown by infrared spectroscopy that the stones did not contain a detectable quantity of calcium bilirubinate. Examination of a stone using the scanning electron microscope showed that most of it consisted of a flaky material having no crystalline habit, and that the interior contained fibrous material and a little crystalline material (fig 5). The crystals were quite large—of the order of 0·1 mm in length. The fibrous mass at the centre of the stone would support the theory of nucleation by entrapment on debris or mucus type material. It has been shown, in rabbits, that the gallbladder epithelial cells secrete increased
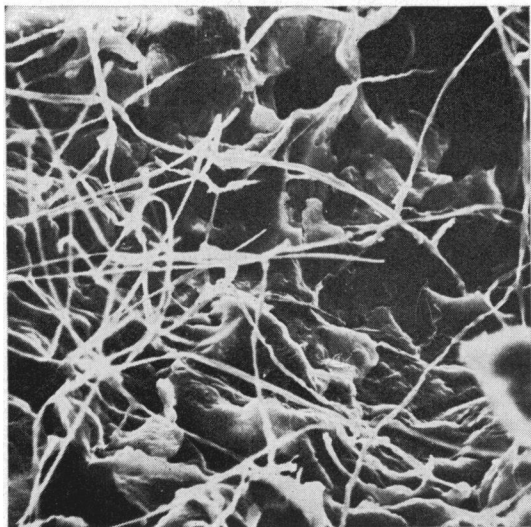
Fig 5a

Fig 5b

Fig 5 *Scanning electron micrographs of gallstone K showing possible nucleation site formed by material trapped in fibres.*

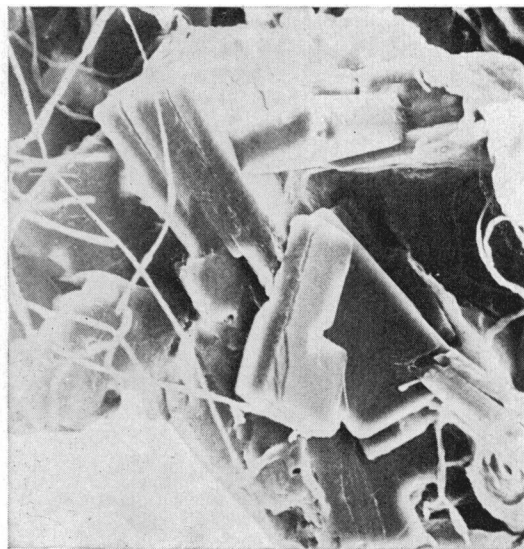Fig 5a *Flaky pigmented material trapped in fibres in stone core.* × 366

Fig 5b *Crystals trapped in the stone core.* × 366

amounts of mucous substances just before gallstones start to form (Freston, Bouchier, and Newman, 1969).

Conclusions

The present study has indicated that pigmented material is always present at the centres of stones, and that the stone centres are often a mass of small crystals and flaky pigmented material trapped in strands of fibres. In general, the theory that nucleation occurs on biliary debris or mucous substances in some gallstones seems to be supported.

Those stones, which consist mostly of cholesterol, can have either a layer structure which has obviously built up step by step around a small centre, or can have a large pigmented central area which has no layer structure but is surrounded by a coating consisting of thin layers of cholesterol. The multistone deposits fall into this latter category and the stones usually contain fibrous material at their centres.

In some cholesterol stones containing calcium carbonate the calcium carbonate is deposited very unevenly, indicating that for some periods during stone growth hardly any calcium carbonate is deposited. Calcium carbonate is often in the form of the rare vaterite, and can be present as columns of small unorientated crystals. The cholesterol in such stones is most likely to be in large orientated flakes radiating from the centre. In cholesterol stones containing fairly evenly spaced layers of calcium carbonate, the cholesterol is unlikely to be present as large flakes, and the calcium carbonate is more likely to be present mainly as aragonite. Calcium carbonate occurs in gallstones in all three of its known crystalline forms and is always associated with pigmented material, although pigmented material can occur without calcium carbonate being present.

The structure of the calcium carbonate present in most cholesterol gallstones is important from the point of view of attempted gallstone dissolution by oral ingestion of compounds such as chenodeoxycholic acid. The presence of calcium carbonate in columns throughout the stone may hinder the solubilization of the cholesterol in the stone. Alternatively, if the cholesterol in such a stone does dissolve, the calcium carbonate structure remaining might act as a nucleus for further stone formation. A cholesterol stone containing isolated regions of calcium carbonate might dissolve rapidly, leaving only small calcium carbonate particles which would be carried away when the bile was expelled from the gallbladder.

It is beyond the scope of this paper to comment on the physiological reasons for the possible changes in bile composition and gallbladder function which

might lead to the precipitation of cholesterol or calcium carbonate, and this aspect is covered by two recent reviews (Bouchier, 1971; Percy-Robb, 1973). However, a detailed knowledge of the structure of gallstones is important because the gallstones contain a record of those changes in the body which influence the function of the liver and the gallbladder. The next stage in the present work is to relate the gallstone structures obtained, using the methods that we have developed, to the case histories of the patients.

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