Electron probe microanalysis in the study of gallstones

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SUMMARY  Detailed information on the structure and composition of gallstones was obtained using an electron probe microanalyser in conjunction with other methods. Gallstones were studied layer by layer without greatly disturbing the arrangement of the materials present. Elements, including trace elements such as copper, iron, and manganese, were identified and their distributions mapped. The range of the method was extended to determine the character and distribution of certain chemical groups present by treating sections of gallstone with reagents which contained easily detected elements. The nature of the bonding of the sulphur in the stones was studied by examining the sulphur x-ray spectrum. Pigmented sulphur-containing deposits were found to contain sulphur in a low valence state but taurine conjugates and the sulphate groups of mucosubstances were not detected. Microcrystalline apatite present in the stones contained some manganese and seemed to be implicated in the adsorption of the low valence sulphur compound and in the nucleation of some stones.

Gallstones have been studied by many methods including x-ray diffraction (Sutor and Wooley, 1971), scanning electron microscopy (Bills and Lewis, 1975; Wolpers and Wosiewitz, 1975), and light microscopy (Nakamura, 1967). These methods, while yielding valuable information, frequently do not show the exact distribution or relative arrangement of different compounds. Studies which show the chemical organisation of intact stones may provide evidence of changes which have occurred in bile composition, including those which resulted in stone nucleation and growth.

In the present work methods are given for the simultaneous determination of stone structure and composition using an electron probe microanalyser. The method is made more versatile by combining it with histochemical and other techniques.

Methods

ELECTRON PROBE MICROANALYSER

The instrument is basically an electron microscope to which is attached equipment for detecting and identifying the characteristic x-rays which are emitted by chemical elements when electrons hit a sample. The usual scanning electron microscope image and the corresponding x-ray images are displayed simultaneously so that the elements can be attributed to known features of the specimen surface.

In the present work a JEOL JXA 50A instrument was used: (1) to identify and locate elements present in the stones; (2) to locate compounds labelled by histochemical means; (3) to identify types of chemical bond; and (4) to electron-excite luminescence as an aid to material identification.

DISTRIBUTION OF ELEMENTS IN GALLSTONES

Distribution maps of calcium, phosphorus, and sulphur were obtained. The position of one element relative to another could be seen and correlated with surface features such as pigmented areas of interfaces between different layers. The detected elements could be assigned to compounds which are known to occur in gallstones (Table 1).

Table 1  Possible compounds of elements found in gallstones

<table>
<thead>
<tr>
<th>Detected elements</th>
<th>Possible compounds</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca</td>
<td>Calcium carbonate, phosphate, bilirubinate, or palmitate</td>
</tr>
<tr>
<td>S</td>
<td>Thiol or disulphide-containing peptide/protein</td>
</tr>
<tr>
<td></td>
<td>Taurine-conjugated bile acid or other</td>
</tr>
<tr>
<td></td>
<td>taurine derivative</td>
</tr>
<tr>
<td></td>
<td>Polysaccharide sulphate ester</td>
</tr>
<tr>
<td></td>
<td>Biliary excreted foreign compound</td>
</tr>
<tr>
<td>P</td>
<td>Amorphous or crystalline inorganic phosphate</td>
</tr>
<tr>
<td></td>
<td>Phospholipid—for example, lecithin</td>
</tr>
</tbody>
</table>

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For rapid element detection an energy dispersive Si-Li detector and multi-channel analyser were used.

**Identification of compounds by histochemical labelling**

Histochemical labelling of gallstone sections using easily detectable elements extends microanalysis from the elemental to the molecular level. Use was made of the standard labelling techniques normally used with light microscopy. However, electron probe analysis has the added advantages of being suitable for pigmented samples and of being able to show the arrangement of naturally contained elements and suitably labelled molecules simultaneously.

Thin sections of stone were chemically treated so that particular molecular groups would react with labels containing elements such as copper or mercury. After reaction, the dried sections were mounted on aluminium foil using double-sided adhesive tape, and vacuum coated with carbon to render them electrically conducting. The histochemical reactions are shown in Table 2. Controls were used to check that the labelling agents were specific in their reactions. For example, groups which were under investigation were blocked by a prior reaction as shown in Table 2. The acidity of many of the reagents caused rapid mineral loss which resulted in fragmentation of some sections. This was avoided by slowly demineralising sections using 5% EDTA (disodium salt) adjusted to pH 7-0 before carrying out the labelling reactions.

For the thiol-labelling reaction, p-chloromercuribenzoate (PCMB) was dissolved in 0.05 M NaOH, the solution adjusted to pH 5-0, and the reagent filtered. Gallstone sections were treated for nine hours at 25°C with the filtrate. Other sections were reacted first with thioglycolic acid to reduce any disulphide bonds within the gallstones, and then were treated similarly.

**Identification of sulphur bond types**

Kα wavelength shifts and Kβ spectral profiles can be used to determine the way in which sulphur is chemically bonded—for example, as sulphate or sulphide. Cysteine, taurocholic acid, and heparin were examined as standards as these are sulphur containing materials of known bond type which resemble components of bile. The sulphur Kα wavelengths and Kβ profiles of several inorganic sulphates and sulphides were also examined. Precautions were taken to reduce beam damage and to obtain accurate wavelength measurements (White, 1973).

The Kα wavelengths of sulphur were measured relative to the third order Kα lines of cobalt, (Co Kα1 = 5.36676 Å and Kα2 = 5.37834 Å).

**Identification of materials by luminescence**

Electron bombardment causes some materials to luminesce. The different luminescence colours of calcium carbonate and cholesterol were observed using a light microscope attached to the microanalyser, and were used to locate these compounds.

**Other methods of analysis**

X-ray diffraction and infrared spectroscopy were used to identify small quantities of gallstone components after microprobe studies had indicated areas of interest. When necessary, cholesterol was removed by extraction with benzene or fragments were demineralised before analysis. Nickel-filtered copper radiation was used for the x-ray diffraction and a Perkin-Elmer 457 instrument for infrared studies. Stone fracture surfaces were examined using a Cambridge mark IIIa scanning electron microscope.

Table 2  Histochemical labelling reactions for compound identification

<table>
<thead>
<tr>
<th>Groups labelled</th>
<th>Labelling agent</th>
<th>Reaction product</th>
<th>Control reaction</th>
<th>X-ray sought</th>
<th>Histochemical reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anionic groups including carboxyl and sulphate ester groups of polysaccharides</td>
<td>Alcian-blue 8GX (section fixed in neutral formalin)</td>
<td>Salt-linked copper phthalocyanine</td>
<td>Prior methylation of acid groups (Nakamura, 1967)</td>
<td>Copper Kα</td>
<td>Pearse (1968) (p. 672)</td>
</tr>
<tr>
<td>Anionic groups including carboxyl and sulphate ester groups of polysaccharides</td>
<td>Colloidal iron (section fixed in neutral formalin)</td>
<td>Electrostatically bound colloid (Prussian Blue reaction omitted)</td>
<td>Prior methylation of acid groups (Nakamura, 1967)</td>
<td>Iron Kα</td>
<td>Pearse (1968) (p. 670)</td>
</tr>
<tr>
<td>Thiol disulphides (eg. from proteins)</td>
<td>p-chloromercuribenzoate (PCMB) before/after reduction with thioglycolate</td>
<td>Mercaptide</td>
<td>Subsequent reaction with cysteine to reverse mercurial binding</td>
<td>Mercury Lα and Lβ</td>
<td>Means and Feeney (1971)</td>
</tr>
</tbody>
</table>
Gallstones Studied

Most of the present work was carried out on stones from the gallbladder of a 46 year old woman. These consisted of 39 faceted, cream-coloured stones composed largely of cholesterol. The stones had surfaces which were smooth to the unaided eye and were classified into two groups according to size. Thirty-six small stones had a mean mass of 0·12 g and a mean diameter of 3·5 mm. The three remaining stones were around 20 times heavier with a mean diameter of 10 mm. The stones also contained calcium carbonate, calcium phosphate, and brown pigment. The smaller stones had black centres, and a similar black pigmentation occurred as a 50 μ thick band near the outer layers of the large stones.

Preparation of Stones for Microanalyser

For the detection of elements naturally present in the gallstones, the friable stones were supported in wax (melting point 57°C) and a smooth surface cut using a microtome. The cut stones were mounted on a conducting base and coated in vacuum with carbon for analysis. New surfaces were cut at small intervals so that a picture was built up of the distribution of elements at different depths.

For the histochemical labelling of compounds, thin sections (8 μ) of stones were cut, after reinforcing using the vinyl acetate film technique of Nakamura (1966). The stone to be sectioned was coated with a thin film of resin, microtomed, and the newly-exposed surface of the section used for analysis. After histochemical treatment, the dried and mounted section was carbon coated and analysed for marker elements.

Results

Elements Present in Gallstones

Calcium, sulphur, and phosphorus distributions were determined in successive layers of the large gallstones. Figure 1 shows the positions of these elements in a section of the same stone.

It can be seen that several calcified bands surround some small calcium rings in the centre of the stone. Examination of adjacent layers of the stone showed that these small rings were sections of spheres. Only the outermost highly calcified band was associated with phosphorus. X-ray diffraction and infrared studies suggested that the sulphur band contained a non-crystalline bilirubin-like component, while regions outside the band consisted largely of cholesterol. Several regions within the sulphur band were found to contain about 2% sulphur when analysed relative to bovine serum albumen/cysteine standards.

X-ray diffraction showed that the phosphorus band contained calcite and microcrystalline calcium hydroxyapatite. Crystal field splitting of an infrared absorption band at 600 cm⁻¹ showed the presence of some crystalline phosphate. Hydroxyapatite cones were distributed around the phosphorus band of all the large stones, and frequently had calcite projecting from the cone bases to the stone centre.

The small stones had black centres which were similar in size to the hydroxyapatite cones of the large stones. The centres of the small stones contained sulphur and large quantities of calcium and phosphorus, suggesting that the cones in the phosphorus band of the large stones and the centres of the small stones had a common origin. In contrast, x-ray and infrared studies showed that the centres of the large stones contained small quantities of cholesterol and calcium bilirubinate, with calcium carbonate in the form of vaterite as the only major inorganic component.

The outer cholesterol layer of the large stones was made up of many thin (100 μ) concentric shells. The innermost surface of this layer contained cavities into

Fig. 1 Distribution of Ca, P, and S in a section of gallstone. (A) Ca x-ray map showing bands which surround a number of central calcified rings. Light areas represent regions of high element-density. Magnification × 50. (B) P x-ray map showing coincidence of a band containing phosphorus with the outermost calcified band of Fig. 1A. A phosphorus cone is present at the bottom of the band. (C) S x-ray map showing a band of sulphur which lies outside and adjacent to the phosphorus band. The locus of increased phosphorus deposition (Fig. 1B) can be seen to be associated with relatively less sulphur than the remainder of the phosphorus band.
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which the hydroxyapatite cones protruded. Manganese was found in small quantities associated with the hydroxyapatite and to a lesser extent with the calcite (Fig. 2). No manganese was detected in the regions containing vaterite. Furthermore, the calcite displayed an intense orange-red luminescence under the electron beam consistent with the presence of manganese-activated emission centres.

![X-ray spectrum showing the presence of manganese within the calcium hydroxyapatite band of a large gallstone.](image1)

The band containing sulphur (Fig. 1) gave fracture surfaces showing some radially oriented texture (Fig. 3), but no evidence of occluded bacteria. X-ray diffraction showed that the sulphur band was non-crystalline. Copper and iron were found in small quantities. The restriction of manganese, copper, and iron to regions containing phosphorus and sulphur in both the small and large stones suggested that these regions had a common origin.

**HISTOCHEMICALLY LABELLED COMPONENTS**

The reactions listed in Table 2 were used to add marker elements to thin gallstone sections in order to label sites containing reactive groups. The distribution of the marker was then mapped using the electron microprobe.

Using sections treated with copper phthalocyanine or colloidal iron, bound yet reactive groups which would include the acidic groups of mucopolysaccharides were found to be diffusely distributed throughout the stones.

Figure 4 shows the distribution of the mercury marker after the test reaction for thiols. The results of treating sections with PCMB before reduction with thioglycolic acid suggested that the sulphur band contained thiols. Treating the sections with PCMB after reduction with thioglycolic acid increased their affinity for mercury and suggested the presence of disulphide groups. The sulphur band had previously been found to contain traces of copper and iron and it was further shown that this band had a high affinity for added cupric ions.

![Distribution of the thiolreactive mercurial within the sulphur band. Magnification × 250.](image2)

The affinity of the sulphur band for colloidal iron and Alcian-blue was low, resembling that of adjacent low-sulphur areas, and indicated that reactive sulphate groups were largely absent. Infrared, melting point and solubility studies also indicated that taurine-conjugated bile acids were not detectable in the sulphur band.
Identification of sulphur bond types

The histochemical work has shown that sulphur in an unoxidised state was present within the sulphur band. Therefore sulphur $K\alpha$ wavelengths and $K\beta$ profiles were examined to confirm the presence of sulphur in a reduced form and to seek other sulphur-containing species.

The organic compounds used as standards gave $K\alpha$ wavelength values which closely resembled those of the more thermostable inorganic standards containing sulphur in a similar valence state. These values correlated well with literature values for related compounds (Sato et al., 1967). It can be seen from Fig. 5 that the sulphur $K\alpha$ wavelength for the sulphur band of the gallstone was close to those of the thiol and inorganic sulphide standards. This was consistent with the evidence from the histochemical labelling studies. It was therefore concluded that taurine conjugates and the sulphate groups of mucosubstances were not present in detectable amounts. Further evidence of the low valence state of the unknown sulphur compound was obtained by oxidising some of the material with performic acid. This resulted in a $K\alpha$ wavelength shift in the direction of sulphur oxidation.

Sulphur $K\beta$ profiles from the organic and inorganic sulphates were associated with a low energy satellite which was displaced from the $K\beta$ peak by about 13 eV. Wilbur and Gofman (1965) observed a similar satellite when sulphur was bonded to two or more oxygen atoms. This satellite was not observed for the $K\beta$ profiles of the inorganic sulphides, the organic thiol or for regions within the sulphur band of the gallstone. These results further indicate that the sulphur-oxygen bonds of taurine conjugates and sulphated mucosubstances were not present as major components of the sulphur band.

Discussion

The electron microprobe has been used to show the arrangement and character of the different chemical phases present in the gallstones examined. Figure 6 showing two sections of the cut surface of a large stone summarises the results. The three large stones contained calcium carbonate as both vaterite and calcite. The formation of vaterite at the centres of these stones and calcite in the outer layers would imply that changes had occurred in the nature of the bile during stone growth. Different sectors of large stones showed similar overall patterns of distribution but with some differences. These differences may reflect the formation of separate parts of a mineral shell within dissimilar environments.

In sector B, the calcified bands lying near to the hydroxyapatite band were composed of discrete calcite deposits, many of which were roughly conical. Without exception, the apices of cones within this calcite ring pointed to the stone centre. Conversely, cone-apices within the next, more central band of calcite pointed towards the stone surface. This arrangement suggests that conditions initially favoured a gradual reduction in carbonate deposition in the inner band which was followed by a gradual increase as the outer ring formed. Calcite frequently projected from the bases of the hydroxyapatite cones, and the deposition of these phases may be related by their formation in the vicinity of a common resorp-

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**Fig. 5** $K\alpha$ wavelength of sulphur within the black band of a large stone in relation to $K\alpha$ wavelengths of known compounds. $a =$ sodium sulphate (inorganic sulphate), $h =$ heparin (organic sulphate), $t =$ taurocholic acid (sulphonate), $c =$ cysteine (thiol), $s =$ gallstone sulphur, $i =$ nickel sulphide (inorganic sulphide).

**Decreasing sulphur oxidation**

![Graph showing decreasing sulphur oxidation](image-url)

**mean sulphur $K\alpha$ wavelength (Å)**

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Fig. 6 Diagrammatic summary of the experimental findings.

tive cavity in the bladder wall or by epitaxy. The innermost calcium bands of sector B showed bulges resembling concentric arcs. The components of each assembly of concentric arcs lay along a radius, suggesting formation by a common process.

Bound yet reactive acidic groups were found to be diffusely distributed throughout the stones. Acidic groups of mucopolysaccharides have frequently been found in gallstones (Maki et al., 1971) and their possible roles in nucleating and aggregating crystalline components of gallstones have been discussed (Freston et al., 1969).

The calcium hydroxyapatite shown in Fig. 6 was in the form of microcrystals, showing that its nucleation was easy, but that growth was difficult. Manganese was associated with the hydroxyapatite and is known to be capable of substituting for calcium in the calcium hydroxyapatite lattice, and of activating phosphatases. Such activation accompanied by the liberation of phosphate ions could lead to the nucleation and growth of hydroxyapatite.

The chemical composition of the centres of the small stones resembled that of the hydroxyapatite cones and the adjacent sulphur band of the large stones, and implied that they formed within chemically similar environments. The dimensions of the cones and the small stone centres, which were similar, suggest that they originated in the resorptive cavities which are believed to exist in the gallbladder wall (Hultén, 1968), and subsequently were released into the bile.

The insoluble sulphur band had the character of a protein-pigment complex. A gallbladder containing hydroxyapatite has a two-fold potential for bringing bile protein molecules into close proximity since fluid is resorbed through the bladder wall, and since hydroxyapatite can adsorb proteins. Copper and iron which were found in the sulphur band could subsequently render adjacent protein molecules insoluble by catalysing the formation of disulphide bonds between them, and promote pigment deposition.

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References


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