THE ANALYSIS OF CALCULI USING MICROCHEMICAL METHODS

BY

P. F. HOLT AND A. L. TÁRNOKY

From the University of Reading and the Royal Berkshire Hospital, Reading

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Microanalytical methods are useful for the analysis of renal and biliary calculi, not only because they are capable of dealing with very small quantities of material, but also because of the ease and elegance with which the analysis can be effected. The scheme described here is derived largely from well-known reactions. It has been designed to use the ordinary equipment and common reagents of a clinical laboratory, although micro-gas reaction vessels are employed and a few tests may be unfamiliar. The methods allow of the separate analysis of thin layers of calculi and can be used to decide the origin of the small quantities of "gravel" sometimes produced by patients or general practitioners.

Experimental Material

Equipment.—The two gas reaction vessels required are micro-test tubes with standard ground-glass joints, closed by small funnels (Vogel, 1945; Briscoe and Holt, 1950). These are available from laboratory furnishers.

Spot tests are carried out on microscope slides (preferably cavity slides) and are observed against a white background. For tests without heating, porcelain spot tiles can be used.

Drops of liquid are delivered from Pasteur capillary pipettes, using rubber teats, or the pipettes and teats of the type supplied with B.D.H. "capillarit" sets.

For tests using filter paper, drop reaction paper (Whatman's No. 120) is the most suitable; except for the alizarin test, Whatman's No. 42 is also satisfactory.

Reagents.—These should be of analytical reagent quality. (1) Acetic acid, glacial; (2) acetic anhydride; (3) ammonia solution, concentrated; (4) ammonium molybdate, 5% (w/v) in water; (5) ammonium thiocyanate solution, such as approximately 0.1 N; (6) chloroform; (7) diphenylamine; (8) ethanol, absolute; (9) ethanol, 70% (v/v) in water; (10) ether; (11) Fouchet's reagent for which 1 ml. aqueous 10% (w/v) ferric chloride solution is added to 10 ml. of a 25% (w/v) solution of trichloroacetic acid in water; (12) hydrochloric acid, approximately 2 N; (13) Nessler's reagent prepared according to the instructions of King (1901) or Harrison (1947); (14) nitric acid, concentrated; (15) nitric acid, approximately 2 N; (16) phosphoric acid, syrup; (17) picric acid, a saturated aqueous solution diluted 1:10 with water; (18) potassium cyanide, a 3% (w/v) solution in water; (19) quinalizarin paper (Briscoe and Holt, 1950), for which 10 mg. quinalizarin is dissolved in 2 ml. pyridine and diluted to 20 ml. with acetone, when Whatman's No. 120 paper is soaked in the solution and then air-dried; (20) sodium acetate solution, approximately 10% (w/v) in water; (21) sodium carbonate, anhydrous; (22) sodium carbonate—phenolphthalein indicator (Vogel, 1945), for which 1 ml. 0.1 N sodium carbonate is mixed with 2 ml. 0.5% (w/v) solution of phenolphthalein in ethanol and with 10 ml. water; (23) sodium hydroxide solution, which is about half-saturated solution of NaOH in water prepared immediately before use; (24) sodium hydroxide solution, approximately 20% (w/v) in water; (25) sodium hydroxide, approximately 2 N; (26) sodium nitroprusside solution prepared when required by dissolving a small crystal in a few drops of water on a slide; (27) Sudan III stain: saturated (approximately 2% w/v) solution of Sudan III in equal vols. acetone and 70% ethanol; (28) sulphuric acid, concentrated.

Methods

Renal Calculi.—The complete scheme is that given in Fig. 1. It will often be found sufficient to test only for ammonia, calcium, carbonate, phosphate, oxalate, and urate.

A small quantity of the stone is powdered, for instance, by placing a portion on a microscope slide and crushing it with a spatula.

Protein.—A small speck of powder is placed on each of two cavity slides. One drop each of 2 N HNO3 is added, and the slides are heated for about 20 seconds over a micro-burner, so that the liquid boils for one to two seconds. The slides are placed on a white background and are allowed to stand for a few minutes. Two drops of 2 N NaOH are then placed on the paler of the two slides. Protein is shown by a marked darkening, giving a definite yellow-brown as compared with the untreated slide.
Indigo.—These stones are rare, and only those which are dark need be tested.

1. To oxidize to isatin, one or two drops of concentrated HNO₃ are added to a small amount of powder on a spot tile. The dark-blue powder dissolves immediately to give a brown solution.

2. To reduce to leuco-indigo, equal volumes (0.1 ml. each) of a dilute solution of the powder in glacial acetic acid and of sodium hydrosulphite solution are mixed in an ignition tube or gas reaction vessel. The solution turns yellow immediately.

3. To reoxidize to indigo, the blue colour returns on heating the solution of leuco-indigo at 100° C. for two minutes.

Fat.—Some of the powder is placed in a gas reaction tube and is shaken with 4 or 5 drops of ether. The powder is allowed to settle, and 3 drops of the ether solution are transferred to a cavity slide. The solution is allowed to evaporate, and the residue is flooded with Sudan III stain. After five minutes the slide is washed with water, 70% ethanol, and, immediately, again with water. Fat appears as a red spot on a clear background. The ether remaining in the gas reaction tube is allowed to evaporate.

Oxalate.—A fragment of the residue is placed in a micro-test tube, and a little diphenylamine is added, followed by 2 drops of syrupy phosphoric acid. The tube is heated directly over a flame for several minutes, and then 5 drops ethanol are added. A blue solution (aniline blue) indicates oxalate. Cystine reacts similarly.

Cystine.—This test need be applied only if the aniline blue test for oxalate is positive. A small fragment of the residue is transferred to a spot tile. One drop of potassium cyanide, then 1 drop of sodium nitroprusside solution are added. A violet colour indicates cystine. This test depends on the formation of an additional compound of sulphide, derived from the cystine, and nitroprusside.

If both the aniline blue and the nitroprusside test are positive, cystine is present, but oxalate may be present or absent. Its presence is indicated if the stone contains either calcium or magnesium and ammonium.

Carbonate.—The residue remaining in the gas reaction tube is used for this test. One drop of carbonate-phenolphthalein indicator is introduced into the capillary of the funnel, 3 drops 2 N HCl are added to the tube, and the funnel is quickly replaced. The tube is mixed by rotating it, and is allowed to stand at room temperature for 15 minutes with occasional mixing. A fading phenolphthalein solution shows the presence of CO₂. Small amounts may cause incomplete fading, with the lower half of the indicator column turning colourless, while the top is still red. The phenolphthalein indicator is carefully removed by means of a teat pipette, and the funnel is taken off.

Magnesium and Aluminium.—One drop of supernatant liquid is applied to quinalizarin paper. A blank of one drop of water is similarly applied. The papers are held over an open ammonia bottle, when the reagent becomes blue. Magnesium gives a bluer and aluminium a redder spot than the blank. A drop of glacial acetic acid is added to each of the spots. This decolorizes the reagent and the magnesium spot, while an aluminium spot turns an even more strongly red violet.

Ammonia.—A drop of Nessler's reagent is placed in the capillary of the other funnel. The contents are gently mixed. The tube is left at room temperature for five minutes. Ammonia produces a yellowish-brown precipitate in the capillary.

Uric Acid and Xanthine.—Two drops of the alkaline supernatant liquid and 1 drop concentrated HNO₃ on each of two cavity slides are heated to dryness on a steam-bath. Uric acid gives a yellowish-red deposit. One drop concentrated NH₄OH is added to one slide. Uric acid gives a red-violet colour. The precipitate is again evaporated to dryness, and 1 drop 20% NaOH is added. Uric acid gives a blue-violet colour.

In the absence of urates, the other slide is heated over a micro-burner. An orange, which changes to red on the addition of 20% NaOH, denotes xanthine.

The contents of the gas reaction tube are acidified by the addition of 4 drops 2 N HNO₃. The solution obtained is used in the tests for calcium, iron, and phosphorus.

Calcium.—One drop of picrolonic acid solution is added to a mixture of the test solution and sodium acetate (1 drop each) on a warm microscope slide. The mixture is left for about 10 minutes and is then
examined under the microscope using a low-power (1/2 in.) objective. Calcium picrolonate crystallizes in long, rectangular plates. (The reagent itself may also give crystals but of a different shape.)

**Iron.**—To 1 drop of the test solution on a cavity slide or spot tile is added 1 drop ammonium thiocyanate solution. Ferrithiocyanate gives an intense red. This reaction is very sensitive (it detects 0.25 μg. Fe++,+) and faint pink reactions should be disregarded.

**Phosphorus.**—Two separate drops of the test solution are placed on a spot tile, and 2 drops 2 N HNO₃ are added to one of these. Ammonium molybdate reagent (3 drops each) is added to both solutions, and the tile is placed in an incubator at 37° C. An intense yellow precipitate due to phosphorus is formed in a few minutes. (The two tests under different conditions are run simultaneously because the one or the other may be the more sensitive, according to the composition of the calculus. Incubating both is preferable to a more elaborate control of the nitric acid concentration.)

**Silica.**—In veterinary pathology and where the origin of the stone is suspect, a part of the calculus is mixed with three or four times its bulk of anhydrous Na₂CO₃. The mixture is taken up in a loop of platinum wire and fused. The melt is dissolved in a few drops of water with heating. The solution is acidified with 2 N HNO₃, and the test for phosphorus is repeated, allowing 20 minutes for incubation. A yellow liquid indicates phosphorus or silica. Some of this is removed with a teat pipette and is placed on drop reaction paper. Phosphorus gives a colourless solution diffusing from a yellow centre. The silica solution forms a uniform yellow spot.

**Biliary Calculi.**—The complete scheme is given in Fig. 2. The tests for protein, fat, and iron will sometimes be omitted.

A portion of the stone is crushed.

![Diagram](http://jcp.bmj.com/)

**Powdered Stone**

- Xanthoproteic reaction
- CHCl₃ extraction
- Molybdic acid

**Extract**

- Sudan III
- Fouchet

**Fat**

- Bile pigment
- Cholesterol
- Carbonate

**Solutions**

- Picrolonate
- Thio-cyanate
- Molybdic acid

**Calcium iron phosphate**

**Protein.**—The test is the same as for renal calculi.

The rest of the powder is dissolved by shaking with a few drops of chloroform in a gas reaction vessel. The solution is allowed to settle. (It can be centrifuged if necessary by standing the gas reaction vessel in a centrifuge tube on a piece of cotton-wool.)

**Fat.**—Two drops of the supernatant liquid are pipetted on to a cavity slide, and the chloroform is allowed to evaporate. The residue is dissolved in two drops of ether and this again is evaporated. The test is then carried out in the same way as in the case of renal calculi.

**Bile Pigment.**—A drop of chloroform solution on drop reaction paper is treated with 1 drop Fouchet's reagent. In the presence of bile pigments (mainly bilirubin) a dark-green reaction develops in a few minutes.

**Cholesterol.**—The Liebermann-Burchard reagent is prepared immediately before the test by mixing 10 drops acetic anhydride with 1 drop H₂SO₄. Two drops of the test solution are placed on a cavity slide or spot tile, and the solution may be diluted with a few drops of chloroform. One drop of the reagent is added. The presence of cholesterol is shown by a yellow colour which changes into blue, or, at higher dilutions, to a greenish blue, in a few minutes. Certain bile pigments give a red turning to a violet-brown. Some bile pigments may be expected in every gallstone. This affects the quality of the colour. Where the presence of cholesterol is in doubt, a drop of the chloroform solution is evaporated on a cavity slide, the slide is flooded with a few drops of nearly boiling ethanol (the hot mixture may be stirred with a teat pipette), and the solvent is again allowed to evaporate. The characteristic notched crystals of cholesterol can be observed under the microscope, using low magnification.

**Carbonate, Calcium, Iron, and Phosphorus.**—The gas reaction tube is heated to evaporate any remaining solvent, is allowed to cool for a few minutes, and the test for carbonate is carried out as for renal stones. After removing the funnel portions of the HCl solution are tested for calcium and iron, again as for renal calculi. The renal calculus test is also applied for phosphate, except that 1 drop 2 N HNO₃ is added to one spot and 3 drops to the other.

**Silica.**—Where it is necessary, this test is carried out in the way described for renal stones.

**Discussion**

The complete analysis of a renal or biliary calculus requires not more than 5 mg. material. The working time involved is certainly not more than would be required by macrochemical methods of analysis.

Some comment is needed on the experimental conditions chosen here for the xanthoproteic reaction for proteins and the murexide test for uric acid and xanthine. While the two reactions are not easily confused when using textbook methods, scaling down these procedures brings...
out their essential similarity. The identification of uric acid offers no difficulties; proteins and xanthine, however, are apt to give the same reactions. Here the strength of the nitric acid has been found to be critical, and 2 N was chosen as a concentration which gives a positive test with 80 μg. protein and is negative with strong solutions of xanthine. Although uric acid may give a yellow spot on treatment with 2 N sodium hydroxide, this will always contain violet parts, usually in streaks.

While this scheme provides an adequate system of analysis for all the common calculi and some of the less common ones, it will not, of course, give a complete answer in all cases. It will not detect xanthine in the presence of uric acid, and may fail to show traces of iron in a predominantly oxalate stone.

Summary

A scheme is presented for the microanalysis of renal and biliary calculi, employing some of the features of microchemical technique while making use of apparatus and methods commonly used in clinical laboratories.

The formal similarity of the xanthoproteic and murexide tests on this scale is emphasized.

References