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12. Goro Chihara,* Sachiko Yamamoto,* and Haruo Kameda**: Medical and Biochemical Application of Infrared Absorption Spectra. I. Studies on Gall Stone by Infrared Spectra and X-Ray Crystallography.

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Clarification of chemical composition of a gall stone would be important for studies on etiology of gall stone formation and for diagnosis and treatment of cholelithiasis. H. Fischer and many other workers studied the chemical composition of gall stones and the stones were found to contain cholesterol, calcium salt of bilirubin, calcium carbonate, and tricalcium diphosphate, as well as other substances.

However, analysis of the stone by chemical means required a great deal of effort and was attended with difficulty that it was almost impossible to analyze each stone immediately after surgical removal. It is usual therefore to assume their components and classify them according to their appearance, location, size, and number.

In recent years, two kinds of apparatus and procedures for physical measurement have developed and these are infrared spectrophotometer and an X-ray spectrophotometer with Geiger counter.

Infrared absorption spectrum is now being widely used for qualitative and quantitative analyses and structural determination. This has also offered a new, rapid and reliable method for the analysis of gall stones. The procedure is simple and a small amount of sample, 1~3 mg., is all that is necessary in giving qualitative and quantitative result of the gall stone component within 30 minutes. Infrared spectral analysis of renal calculi has been reported[1] but its application to gall stones has not been made yet.

Analysis of gall stones by the Debye-Scherrer's X-ray powder photography has already been made,[2,3] but a far more rapid and reliable analysis is possible by the use of a recently developed X-ray spectrophotometer attached with a Geiger counter.

The above two methods have advantages and disadvantages and it is desirable that they be used concurrently in accordance with the situation. The rapidity and reliability of modern apparatus are extremely effective when utilized in a clinical laboratory.

The present workers carried out the analyses of over 100 pieces of gall stone by infrared absorption spectrum and X-ray diffraction, established the analytical procedures, and obtained many interesting observations, a part of which are described herein.

I. Infrared Absorption Spectrum

Experimental Method

Apparatus: Hilger Model H 800 automatic recording, infrared spectrophotometer, with NaCl prism, was used to measure the region of 4000~650 cm^{-1}. Wave numbers were corrected with polystyrene.

Materials: Over 100 gall stones obtained by surgical operation in the Department of Surgery, Tokyo University Hospital, were submitted to the analysis.

Experimental Method: The spectra were measured as Nujol mull and KBr disk. A large-

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1) D.E. Beischer: J. Urology, **73**, 653(1955).
2) W. Epprecht, H. Rosenmund, H. Schinz: Fortschr. Gebiete Röntgenstrahlen, **79**, 1(1953).
3) H. Miyake: Saishin Igaku, **3**, 456(1948).

sized gall stone was separated into external, median, and central parts, and a small-sized one was mixed into one sample. For one measurement, 1~3 mg. was used.

Results and Discussions

The infrared absorption spectral data of gall stones obtained by the foregoing method revealed that the majority of gall stones could be classified into following six kinds: Cholesterol stone (Fig. 1), cholesterol-$CaCO_3$ stone (Fig. 2), cholesterol-bilirubin Ca stone (Fig. 3), cholesterol-bilirubin Ca-$CaCO_3$ stone (Fig. 4), bilirubin Ca-$CaCO_3$ stone (Fig. 5), and bilirubin Ca stone (Fig. 6). Figs. 1~6 show the infrared absorption of these stones together with the spectrum of the pure component. It is seen that their chief components are cholesterol, bilirubin Ca, and $CaCO_3$. It was also found that two isomorphic forms of $CaCO_3$, calcite type and aragonite type, are present but this point will be described later.

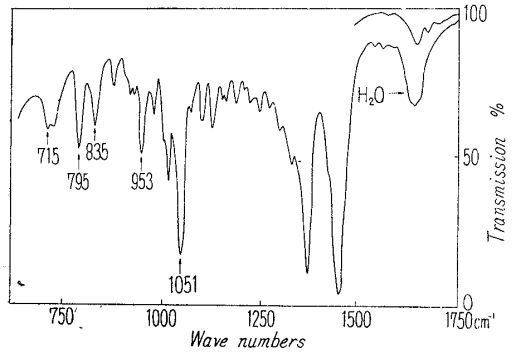

Fig. 1. Cholesterol Stone

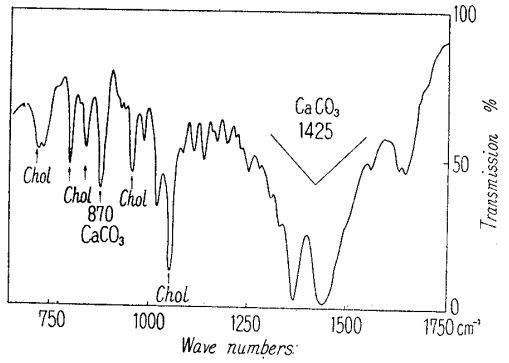

Fig. 2. Cholesterol-Calcium Carbonate Stone

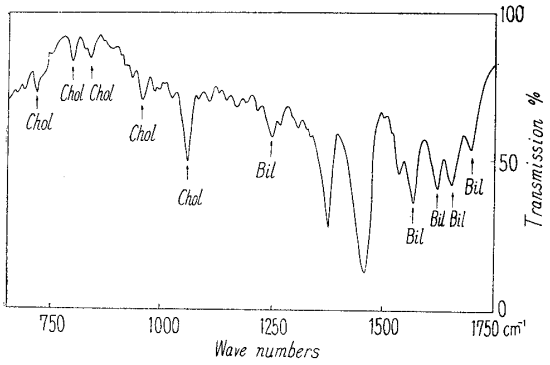

Fig. 3. Cholesterol-Bilirubin Calcium Stone

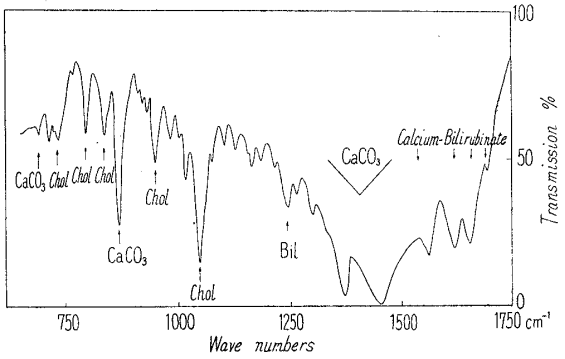

Fig. 4. Cholesterol-Bilirubin Calcium-Calcium Carbonate Stone

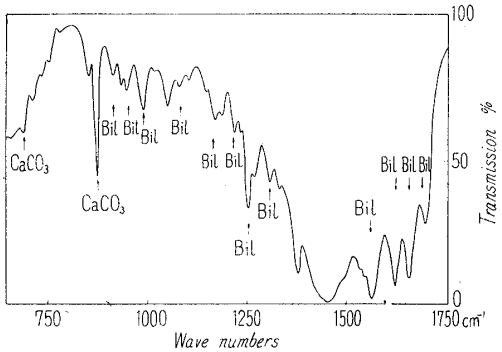

Fig. 5. Bilirubin Calcium-Calcium Carbonate Stone

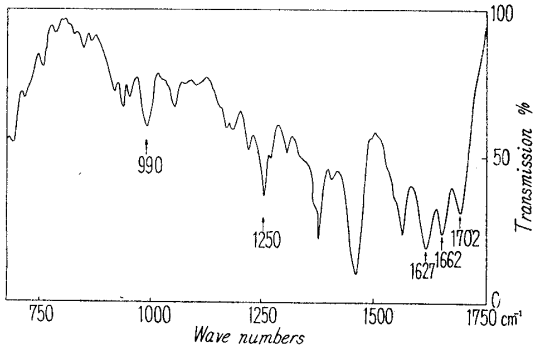

Fig. 6. Bilirubin Calcium Stone

The infrared spectrum of a gall stone is represented as the overlapped spectra of its constitutional chemical components. If a suitable key band is selected, qualitative and quantitative analyses of its components become possible. For the identification of the component, requisite absorptions are given in Table I.

TABLE I.

	Wave number (cm^{-1})
Cholesterol	1052±1, 952±1, 836±1, 795±1, 728±1
Bilirubin Ca	1702±2, 1662±2, 1627±1, 1250, 1186±1, 990
$CaCO_3$ (calcite type)	1410~1450 (1425, strong and broad), 874, 712
$CaCO_3$ (aragonite type)	1420~1480 (1470, strong and broad), 860, 712, 700

For the identification of components, all the absorptions given in Table I must be observed. The absorption of strong intensity at 1052 cm^{-1} is that of cholesterol but this is an absorption of C–OH group and is not necessarily characteristic to cholesterol alone. The broad absorption of strong intensity in the region of 1410~1480 cm^{-1} is that of a carbonate and is not characteristic of $CaCO_3$. The frequencies given in Table I were selected after taking these points into consideration and may be considered as characteristic to these substances.

These infrared spectral analyses have shown that judgement of gall stones from their appearance alone gave very erroneous idea of their components.

The foregoing data are for the chief components but gall stones usually contain many other components. Infrared spectral data were found to offer easy detection and presumption of such substances without going through tedious systematic analysis. Some considerations were made on new and rare components and an example of cholesterol–$Ca_3(PO)_2$ stone is given in Fig. 7 by its infrared absorption spectrum. The strong absorption in the region of 1000~1200 cm^{-1} is that due to phosphate ion.

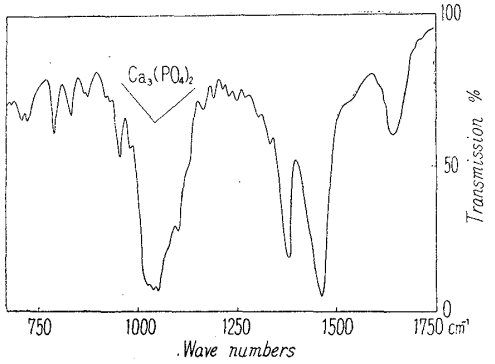

Fig. 7. Cholesterol-Tricalcium Diphosphate Stone

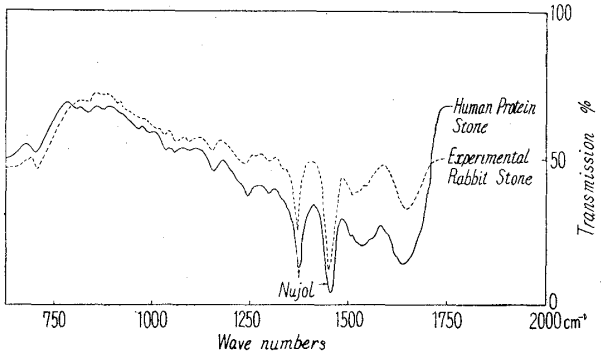

Fig. 8. Protein Stone in Human and Experimental Rabbit

Fig. 8 is the absorption curve of a gall stone artificially produced in a rabbit by injection of serum, in accordance with the theory of allergic formation of a gall stone.

This curve shows a characteristic absorption for proteins and such absorption curve was found in several cases of human gall stone. From its appearance, this kind of a gall stone is identical with that of bilirubin calcium type and had been determined as such. Therefore, the use of rapid infrared spectral analysis would be the most suitable in such cases, and would also prove very effective in clinical diagnosis, therapeusis, and prevention of recurrence.

It was found, as mentioned earlier, that there are two forms of $CaCO_3$ present in the gall stone, the calcite (hexagonal) and aragonite (rhombic). The ratio of the two in the gall stone is varied but in many cases, aragonite alone was found to be present. This is more certain from X-ray diffraction but the two can be distinguished clearly

even by infrared absorption.[4] The frequencies are given in Table I and the absorption curve is shown in Fig. 9. Fig. 10 is the absorption spectrum of the actual gall stone containing both with cholesterol.

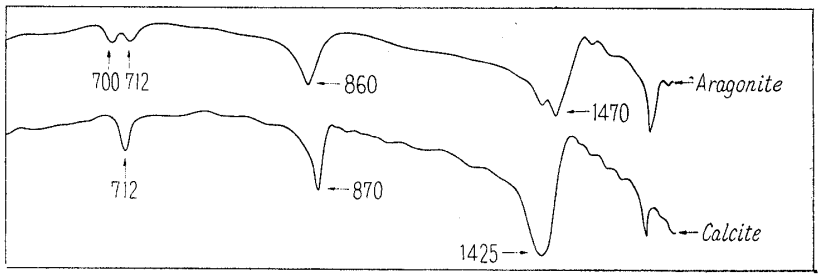

Fig. 9. Infrared Spectra of Aragonite and Calcite

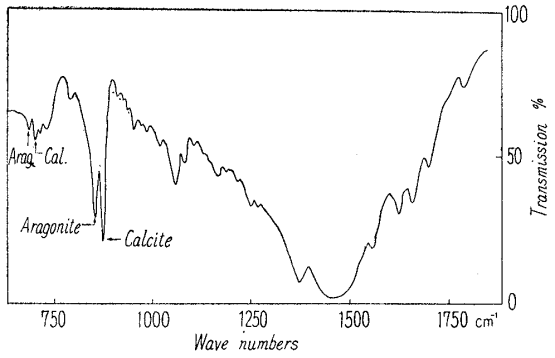

Fig. 10. Aragonite and Calcite Stone (+Bilirubin Calcium)

X-Ray structural analysis of these two types of $CaCO_3$ has been made by Bragg.[5,6] In the calcite, three oxygen atoms are in the same plane, symmetrically situated relative to the carbon atom and in proximity to the two calcium atoms. In the aragonite, three oxygen atoms in the same manner are in close proximity of three calcium atoms and the distance of C–O of 1.24 Å is somewhat different. Therefore, the absorption of degenerated calcite at 712 cm^{-1} is assumed to have split into absorptions of 712 and 700 cm^{-1}.

Aragonite is almost non-existent in mineral world except in mineral spring and is also rare in animal world, the only known examples being in mollusks like the mother-of-pearl. Its appearance in the animal world is probably related to the breeding season and the formation of aragonite is affected greatly by hydrogen ion concentration. Many theories have been forwarded in the past for the formation of aragonite,[7] such as partial pressure of CO_2, the precence of Na^+, K^+, and NH_4^+ cations together, and dilute solutions, and this must be an interesting problem in clinical biochemistry, diagnosis, and therapeusis of gall stone.

II. X-Ray Diffraction

Experimental

Apparatus : Norelico X-ray spectrophotometer with Geiger counter was used and Cu-K_α line was adopted.

Material : The same samples as for the infrared spectral analyses (Figs. 1~6) were used besides natural aragonite and calcite.

Results and Discussion

Similar to the infrared absorption spectra, the X-ray diffraction pattern of the

4) Cray Mineral Standard (API)
5) W.L. Bragg : Proc. Roy. Soc., **A 89**, 468(1914).
6) W.L. Bragg : *Ibid.*, **A 105**, 16(1924).
7) Y. Kitano, M. Nishimura : Nippon Kagaku Zasshi, **76**, 581(1955).

gall stones appears as the overlapping of each component, so that the suitable selection of a key band will make it possible to analyze the components, both qualitatively and quantitatively. However, this procedure is not applicable for amorphous substances like bilirubin calcium and proteins, while this enables a more distinct discrimination to be made between aragonite and calcite. It therefore follows that the coordinate use of infrared and X-rays would be the most advantageous method.

The X-ray diffraction pattern of a gall stone in which aragonite is mixed with cholesterol is shown in Fig. 11.

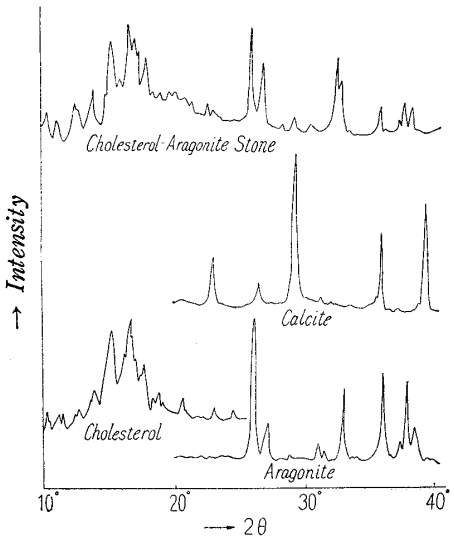

Fig. 11. X-Ray Diffraction Pattern of Cholesterol-Aragonite Stone

Both of these procedures are not suited for the detection of a minute amount of a substance and in such a case, the use of flame analysis would be desirable for the detection of metal ions.

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Summary

1) A rapid qualitative and semi-quantitative analytical procedure for gall stones by infrared absorption spectra was established, and bands for the chief components of the gall stone, cholesterol, bilirubin calcium, and calcium carbonate were clarified.

2) The component of the gall stone, formed artificially in a rabbit by the injection of serum, was found to be a protein and such a gall stone was found in a few cases of human specimen.

3) The calcium carbonate found in gall stones came in two types, argonite and calcite.

4) This procedure was found to be advantageous when employed in a clinical laboratory.

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