UDC 615.362.357-07 : 543.422.4

196. Goro Chihara, Kimiko Matsuo (née Shirakuma), Kumiko Arimoto, and Shigeko Sugano (née Fujii) : Medical and Biochemical Application of Infrared Spectroscopy. VII. Studies on Various Animal Bile by Infrared Spectra and Some of its Pharmaceutical Application, Including Identification of the Bear Bile (Yūtan).

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In the preceding paper of this series,[1] the authors have shown that the application of infrared spectral analysis of human bile was a simple and effective method in the detection of abnormal bile and in knowing changes and variation of bile components. It was proved through this work that the main constituent of bile acids contained in normal human bile is cholic acid and not deoxycholic acid. This work was extended to the studies of bile in various animals.

The present series of work was undertaken for the following three purposes. The first is the identification of Yūtan (熊胆), the dried gall bladder and bile of various bear (*Ursus arctos, Selenarctos thibetanus*), which is a very expensive home remedy (stomachic and tonic) used from olden times in the Orient (Japan, China, India, etc.); the second is re-examination of bile components of various animals; and the third is collecting fundamental data necessary for the study of the pharmacological action of various choleretics, using dogs and rabbits.[2]

Infrared spectra of bile from 18 kinds of animals were examined, including, bear, bovine, sheep, monkey, swine, dog, rabbit, rat, chicken, snake, toad, carp, catfish, eel, sea bream, and flatfish. These spectra were compared with the infrared spectra of various bile components and significant results for the above purposes were obtained.

Experimental

Material——Fresh gall bladder and bile were collected, immediately after slaughter from bovine (*Bos taurus*), swine (*Sus scrofa*), sheep (*Ovis aries*), chicken (*Gallus domesticus*), snake (*Agkistrodon halys, Trimeresurus flaviridis*, and *Elaphe guadrivirgata*), and eel (*Anguilla japonica*), or by operation from dog, rabbit, and toad (*Bufo vulgaris*). The material was immediately dried and reduced to a powder to measure the infrared spectrum. Spectra were also taken of liver bile in the case of rat, dog, and rabbit. In the case of fish (*Crysophrys major, Kareius bicoloratus, Monacanthus cirrhifer*, and *Platycephalus indicus*), gall bladder and bile were collected from the fresh fish on the market.

Dried gall bladder and bile were from the domestic bear (*Ursus arctos* and *Selenarctos thibetanus*) and the one imported from Tibet through India. Bile was collected from a monkey (*Macaca irus*) immediately after slaughter of the one used for manufacture of polio vaccine.

Infrared spectra of each bile were measured 3～50 times. Bile spectra of animals of the same species showed approximately definite shape and it seemed almost unnecessary to consider individual difference in each animal.

Conjugate bile acids used as standard samples for comparison were not isolated from natural source but were synthesized according to the method of Bergstrom and Norman[3,4] from cholic acid, deoxycholic acid, and ursodeoxycholic acid of known purity.

Method——Bile was allowed to stand in a vacuum desiccator by which it dried and reduced to a powder. This was mixed thoroughly and infrared spectrum was measured by the KBr pellet method. About 0.5 cc. of the bile was sufficient for the measurement. Spectra were measured with the Hilger Model H-800 spectrophotometer with NaCl prism, in the region of 2000～650 cm^{-1}.

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1) Part VI. G. Chihara, *et al.* : This Bulletin, **10**, 1184 (1962).
2) G. Chihara, *et al.* : *Ibid.*, **9**, 939 (1961).
3) S. Bergstrom, A. Norman : Acta Chem. Scand., **7**, 1126 (1953).
4) A. Norman : Arkiv Kemi, 8, 331 (1955).

Results and Discussion

Infrared spectra of bile from various animals and those of conjugate bile acid salts are shown in Figs. 1~4. Bile juice of various animals contains smaller amount of mucic substances (proteins, polysaccharides) than human bile and their infrared spectra are affected by the absorption of bile acids and lipids. Consequently, it is not necessary, as was the case in human bile, to extract the bile acid fraction with methanol in order to examine bile acid components.

I. Infrared Spectra of Various Mammalian Bile, especially on the Identification of Yū-tan (Bear Bile)

There has been no suitable method to discriminate bear bile from the bile of bovine, swine, and other animals. However, bear bile contains tauroursodeoxycholic acid as the specific bile acid[5] and it is possible to identify bear bile by infrared spectrum, simply and accurately.

The infrared spectrum (Fig. 1-A) of dried bear bile exhibits absorptions at 1643 (vs), 1543 (vs), 1451 (s), 1419 (w), 1376 (s), 1332 (w), 1309 (w), 1216 (vs), 1173 (w), 1118 (m), 1075 (m), 1045 (vs), 1012 (w), 958 (m), 943 (w), 927 (w), 902 (m), 864 (m), 801 (m), and 742 (m) cm^{-1}. In these absorptions, the absorptions of synthesized sodium tauroursodeoxycholate (Fig. 1-B) are all observed. Absorptions below 1012 cm^{-1} are especially effective for identification and can be used as the key band in the discrimination of bear bile from that of other animals.

Infrared spectrum of bile from animals having taurocholate (Fig. 1-D) as the chief component, such as the bile of bovine, sheep, and dog (Figs. 1-C, 1-E, Fig. 2-I), has absorptions at 980 (m), 948 (m), 924 (shoulder), 917 (m), 901 (w), 859 (m), and 742 (w) cm^{-1} in this region and the position of these absorptions is clearly different from that of bear bile.

Infrared spectra of bovine and sheep bile can be interpreted by the method reported earlier for analysis of bile acids in human bile.[1] According to this interpretation, the main constituent of bovine bile is taurocholate, with some glycocholate (1608, 1403, and 1312 cm^{-1}), and very little of other components. Absorption of lipids (1740 cm^{-1}) is very weak or is absent in some cases (same in the case of bear bile).

Bile acids from sheep bile are the same as in the case of bovine bile with slightly stronger absorption of lipids.

Infrared spectrum of total lipids extracted from bovine and swine bile agrees approximately with that of lecithin, similar to the case of human bile.[1]

Infrared spectrum of swine bile shows characteristic absorptions with absorption bands at 1643 (s), 1604 (vs), 1550 (shoulder), 1453 (m), 1403 (vs), 1313 (m), 1254 (w), 1230 (w), 1200 (w), 1171 (m), 1131 (w), 1110 (w), 1081 (w), 1043 (s), 999 (w), 985 (w), 950 (w), 914 (m), 897 (w) cm^{-1} (Fig. 2-J). As has already been revealed, swine bile contains characteristic bile acids like hyodeoxycholic acid[6,7] and these absorptions are considered to be due to its absorption. Conjugate amino acid is glycine.

Infrared spectrum of rabbit bile[8] has absorptions at 1604 (vs), 1553 (shoulder), 1454 (m), 1411 (s), 1341 (w), 1312 (m), 1259 (m), 1171 (m), 1113 (w), 1098 (m), 1070 (m), 1046 (vs), 1014 (m), 970 (m), 948 (m), 920 (m), 856 (m), 815 (w), 800 (w), and 759 (w) cm^{-1}. These absorptions agree well with those of synthesized sodium glycocholate. Consequently, the main con-

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6) G. A. D. Haselwood : Biochem. J, **56**, xxxviii (1954).
7) *Idem* : *Ibid.*, **62**, 637 (1956).
8) S. Okamura, T. Okamura : Z. Physiol. Chem., **188**, 11 (1930).

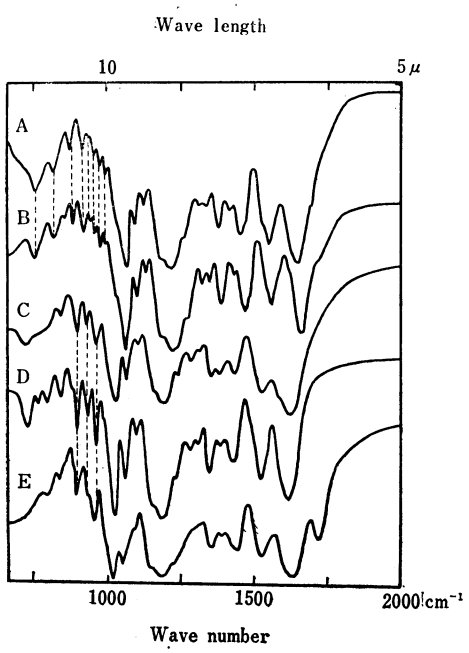

Fig. 1. A) Bear B) Sodium tauroursocholate C) Bovine D) Sodium taurocholate E) Dog

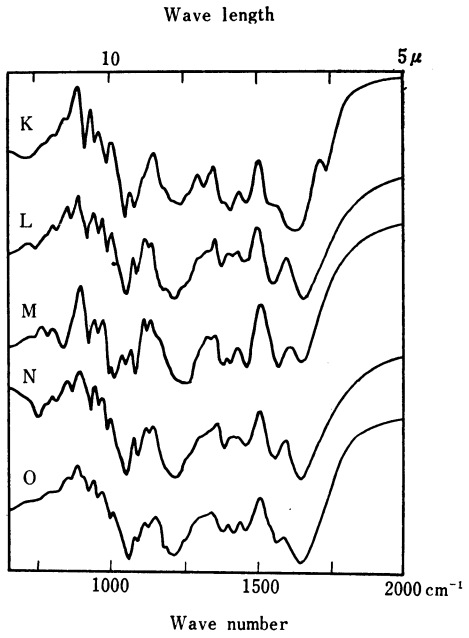

Fig. 3. K) Monkey (*Macaca irus*) L) Snake M) Toad N) Eel (*Anguilla japonica*) O) Catfish (*Parasilurus asotus*)

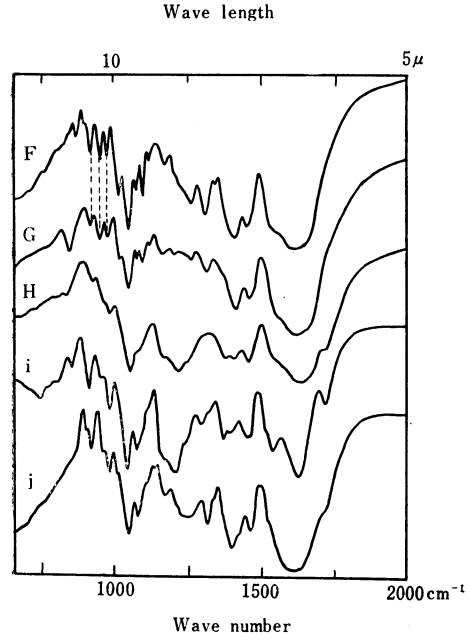

Fig. 2. F) Rabbit G) Sodium glycodeoxycholate H) Rat I) Sheep J) Swine

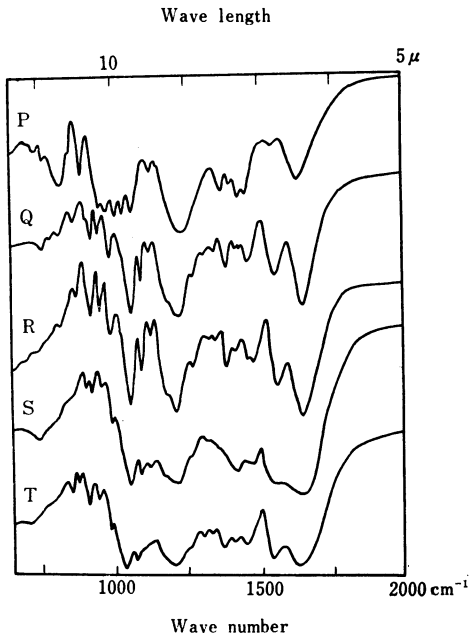

Fig. 4. P) Carp (*Cyprinuscarpio*) Q) Flatfish (*Kareivs bicoloratus*) R) Sea bream (*Crysophrys major*) S) *Monacanthus cirrhifer* T) Flathead (*Platycephalus indicus*)

Figs. 1~4. Infrared spectra of Various Animal Bile and Conjugated Bile Salts

stituent of rabbit bile is glycocholate and other bile acids are probably in a very small amount (Figs. 2-F and 2-G).

There are various reports on the component of monkey bile[9~11] but none on that from *Macaca irus*. The bile from this monkey was found to have glycocholate and taurocholate as the main components, as in the case of human bile, from its infrared spectrum (Fig. 3-K). Ratio of these two components vary greatly, unlike the case of human bile. Absorption bands are at 1747(s), 1734(m), 1650(vs), 1614(vs), 1563(s), 1470 (m), 1410(s), 1388(m), 1347(w), 1318(m), 1264(shoulder), 1250(w), 1229(m), 1208(m), 1170 (w), 1119(shoulder), 1079(s), 1048(s), 978(m), 948(m), 924(m), and 859(m) cm^{-1}.

By the use of these spectra, a large number of Yūtan (dried bear bile) imported into Japan and on the market were examined and variety of counterfeit articles were found, showing absorptions of bovine, swine, monkey, and plant products.

II. Infrared Spectra of Bile Juice from Various Fish, Amphibians, and Reptiles, especially on the Bile Components from Carp

As above mentioned, infrared spectrum is an effective means in the re-examination of bile components of various animals and especially interesting observations were gained with carp bile. Main absorptions in the infrared spectrum of carp bile (Fig. 4-P) are present at 1681(s), 1462(m), 1442(m), 1412(w), 1375(w), 1213(vs), 1122(w), 1062(m), 1031 (m), 1008(m), 981(m), 890(shoulder), 828(s), 808(w), and 738(w) cm^{-1}.

These absorptions are markedly different from the infrared spectra of bile having conjugated bile acid as the main component, such as thos efrom bovine, swine, eel, and flatfish, lacking in the absorptions characteristic to conjugated bile salts such as the amide (around 1650 and 1540 cm^{-1}), carboxylate (1600 and around 1400 cm^{-1}), and free carboxylic acid (around 1730 cm^{-1}). In their stead, strong absorption bands showing characteristics of a sulfate[12,13] are present at 1213, 1062, 878, and 738 cm^{-1}. From the characteristics of the spectrum as a whole, the main component of carp bile is considered to be the sulfate of bile alcohol (cyprinol)[14] and the past report[15] that it is taurocholic acid should be corrected, since there is hardly any cholic acid in carp bile. This is an exception as a teleost.

The component of toad bile has been reported as the sulfate of bile alcohol[16,17] and the infrared spectrum of toad bile was found to be very similar to that of carp bile (Fig. 3-M). However, absorption bands are in slightly different positions from those of carp bile and are at 1654(s), 1635(s), 1563(vs), 1462(s), 1409(s), 1371(m), 1260~1215 (vs), 1123(w), 1077(s), 1045(m), 1004(s), 982(m), 951(w), 919(m), 824(w), 796(w), and 781 (w) cm^{-1}. It is clear from the position of these absorptions that the bile alcohol in carp bile is different from tetrahydroxynorbufostane and pentahydroxybufostane in toad bile.

Infrared spectrum of snake bile, as far as the bile from the snakes examined (*Agkistrodon halys*, *Trimeresurus flavoviridis*, and *Elaphe quadrivirgata*) is concerned, seem to have taurocholate as the main component (Fig. 3-L) and the spectra were no different among these three species. The strong absorption of a carboxylate suggests

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10) H. B. Wiggins : Biochem. J., **56**, xxxix (1954).
11) F. Beck : Proc. Soc. Exptl. Biol. Med., **43**, 603 (1940).
12) G. Chihara : This Bulletin, **6**, 117 (1958).
13) *Idem* : *Ibid.*, **8**, 988 (1960).
14) G. A. D. Haslewood : Biochem. J., **59**, xi (1956).
15) T. Hatakeyama, T. Okamura : J. Biochem. (Tokyo), **9**, 333 (1928).
16) H. Makino : Z. Physiol. Chem., **220**, 49 (1933).
17) T. Kazuno : *Ibid.*, **266**, 11 (1940).

the presence of a small quantity of glycocholate and this is similar to the case of eel and catfish (Figs. 3-N and 3-O).

On the other hand, infrared spectra of bile from *Kareius bicoloratus* and *Crysophrys major* were almost identical with the absorption of sodium taurocholate and other components, even if any, would be in a very minute amount (Figs. 4-Q and 4-R). This is the same in the case of flathead (*Platycephalus indicus*) (Fig. 4-**T**).

Infrared spectrum of *Monacanthus cirrhifer* (Fig. 4-S) was different from those of *Crysophrys major* or of eel and its main component is not taurocholate. The absorption of cholate is present though weak in intensity and a stronger absorption is present in other positions (1097, 900, and 858 cm^{-1}). Since these positions are the same as the absorptions in the spectrum of chicken bile, these absorptions are thought to be due to chenocholate. Conjugate amino acid in the bile acids of teleosts is in general, taurin but that in *Monacanthus cirrhifer* and *Hexagrammos otakii* is probably glycine since their infrared spectra show strong absorptions at around 1640 and 1408 cm^{-1}.

III. Infrared Spectrum of Dog Bile (as a Basis for Examining Pharmacological Action of Various Choleretics)

Dog bile contains only a small amount of mucic substances and the conjugate amino acid is taurin alone.[18,19] Infrared spectrum of dog bile (Fig. 1-E) is very clear and was approximately definite in shape when examined on almost 100 dogs, indicating that there is no necessity to consider individual difference. Absorption bands are at 1740 (s), 1652 (vs), 1547 (s), 1463 (m), 1450 (m), 1411 (vw), 1375 (m), 1336 (w), 1302 (w), 1254 (shoulder), 1208 (s), 1174 (s), 1075 (s), 1045 (s), 978 (w), 952 (m), 913 (m), and 805 (m) cm^{-1}. Dog bile was also collected every 30 minutes for 7 hours but no change in the infrared spectrum was observed.[1]

This measurement was made as the basis for examining the pharmacological action of various choleretics and the dog bile was treated as fort he interpretation of the infrared spectrum of human bile.[1] Dog liver bile, differing from that of human bile, is almost free of mucic substance and the infrared spectrum of total lipids agrees approximately with that of lecithin, as in the case of human beings. Infrared spectrum of bile acids is almost the same as that of sodium taurocholate and absorption of glycocholate is absent. The spectrum of dog liver bile may be interpreted as the overlapped absorptions of lecithin and sodium taurocholate. Absorption of lecithin in dog bile was far stronger than that of bile acids from any of the animals examined in this work. The absorption at 1740 cm^{-1} is effective as the key band for lipids and that below 1000 cm^{-1} as the key band for indicating the quantity of cholic acid.

Since the infrared spectrum of dog bile is very distinct, it is most suitable for examining pharmacological action of various choleretics. The present authors have obtained many interesting observations in this field by the use of a dog[2] and detailed report on this work will be published in the near future, together with the method for collecting liver bile from a dog, which was ommited from the present paper.

The authors are indebted to Messrs. Z. Matsui and K. Ikeda for giving them a large number of bear bile, and to Prof. S. Hara, Prof. K. Yamazaki, and Dr. K. Takeda for the donation of materials for the syntheses of conjugate bile acids. A part of expenses for the present work was defrayed by the Asahi Science Fund.

Summary

A large number of bile from 18 kinds of animals were submitted to infrared spectral measurement and it was revealed that these spectra were very effective in knowing

18) O. Hammerstein : Z. Physiol. Chem., **43**, 109 (1904).
19) G. Saba : J. Biochem. (Tokyo), **30**, 55 (1939).

various bile components and constituents of bile acids. This method was successfully utilized in discriminating Yūtan (bear bile), used as the valuable home remedy in the Orient from olden times, from the bile of other animals.

Some results different from past reports were obtained; notably that the main component of carp bile is not bile acid but is a sulfate of bile alcohol and that the conjugate amino acid in the bile acids of some fish was not taurin.

Infrared spectra of bile from about 100 dogs were examined as basis for the study of pharmacological action of choleretics. Infrared spectrum of dog bile is especially distinct, there is no necessity of considering individual differences, and there are no variation in the amount of bile outflow or infrared spectrum even eight hours after the operation.

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197. Tameto Okanishi, Akira Akahori, and Fumio Yasuda : Studies on the Steroidal Components of Domestic Plants. XL.*1 Constituents of *Heloniopsis orientalis* (THUNB.) C. TANAKA. (3). The Structure of Heloniogenin.

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As previously reported,[1] five steroidal sapogenins were isolated from "Shōjō-bakama" *Heloniopsis orientalis* (THUNB.) C. TANAKA. They are $\Delta^{3,5}$-desoxytigogenin, diosgenin, gentrogenin, kryptogenin and a new sapogenin. This new sapogenin (I), m.p. 212~213°, $[\alpha]_D$ −91°, has two hydroxyl groups. Its ultraviolet spectrum showed an absorption maximum (log ε 3.47) at 205.6 mμ. The analytical values also indicated that this sapogenin has a double bond. The infrared spectrum showed that this sapogenin has no carbonyl function and belongs to 25-D series of the steroidal sapogenins. This sapogenin was readily acetylated to give a diacetate (II), m.p. 184~185°, $[\alpha]_D$ −58°, with pyridine and acetic anhydride under usual conditions and it is considered that two hydroxyl groups are both primary or secondary. Although the known 25D-spirostenediols are yuccagenin[2] (25D-spirost-5-ene-2α,3β-diol), pennogenin[3] (25D-spirost-5-ene-3β,17-diol), ruscogenin[4] (25D-spirost-5-ene-1β,3β-diol) and isochiapagenin[5] (25D-spirost-5-ene-3β,12β-diol), the physical constants of this new sapogenin and its acetate do not coincide with any of those sapogenins and their acetates. From these results it is considered that this sapogenin is a new sapogenin and the name heloniogenin was given to this compound.

Heloniogenin yielded 3-acetate (III), m.p. 218~219°, $[\alpha]_D$ −89.6°, when it was kept at 10° with pyridine and acetic anhydride. The chromic acid oxidation of heloniogenin in acetic acid yielded gentrogenin (IVa) (3β-hydroxy-25D-spirost-5-ene-12-one) with

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*1 Part XXXIX A. Akahori : Ann Repts. shionogi Research Lab., **11**, 97 (1961)

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2) R.E. Marker, *et al.* : J. Am. Chem. Soc., **65**, 1201 (1943); *ibid.*, **69**, 2189 (1947).
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