Cela nous suggère de même que le métabolisme du *P. islandicum* est très semblable à celui des bactéries. Nous considérons que c'est une des plus importantes questions pour que nous puission continuer nos recherches sur le mécanisme biosybthétique de la lutéoskyrine dans la bonne direction.

Résumé

Quant au métabolisme du *P. islandicum*, nous avons essayé de savoir quel est le meilleur processus de l'activation d'acétate, le système de la production de NADPH et la précurseur le plus élémentaire des pigments. Nous avons trouvé : (1) que l'acétokinase activait l'acétate, (2) que la réaction concernant la G-6-P déhydrogénase pourrait produire le NADPH et (3) que la dose incorporée des précurseurs [^{14}C] dans la lutéoskyrine, (un des pigments obtenus des substances métaboliques du *P. islandicum* Sopp) augmentait plus spécialement quand on avait utilisé le sucrose [U-^{14}C] comme précurseur.

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72. Shoji Hara, Michiko Takeuchi, Misako Tachibana,*1 and Goro Chihara*2 : Systematic Analysis of Steroids. IV.*3 Thin-layer Chromatography and Densitometry of Bile Components.*4

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There are numerous works on the components of bile, besides their analysis by infrared absorption spectra.[1] Result of these studies revealed that the chief components are conjugate acids and that there are small or minute amounts of free bile acids, bile pigments, lipids, mucoids, and inorganic substances. However, rapid and reliable methods for separatory determination of each of these components, especially conjugated bile acids, have not yet been available.

Among many techniques devised for such separatory determination, the recently developed thin-layer chromatography seems to be the most useful. In recent years, Gänshirt, *et al.*[2] and Hofmann*5,[3] reported separation of conjugated bile acids in human bile by thin-layer chromatography of pure products and determination by extraction with 65% sulfuric acid, although these experiments were made only with few kinds of pure substances and not directly on natural bile.

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*3 Part II : This Bulletin, **11**, 1189 (1963); Part III : J. Chromatog., **11**, 565 (1963).
*4 A part of this work was reported at the Kanto Local Meeting of the Japanese Biochemical Society, December, 1962; Kanto Local Meeting of the Pharmaceutical Society of Japan, November, 1962, and 83rd Annual Meeting of the Pharmaceutical Society of Japan, November, 1963.
*5 Usui has recently attempted this method (J. Biochem. (Tokyo), **54**, 283 (1963)).
1) G. Chihara, *et al.* : This Bulletin, **10**, 1184, 1190 (1962).
2) H. Gänshirt, F. W. Koss, K. Morianz : Arzneimittel-Forsch., **10**, 943 (1960).
3) A. F. Hofmann : J. Lipid Res., **3**, 110 (1962).

One of the authors (S. H.) has already reported on systematic analysis of steroidal hormones,[4,5] steroidal sapogenins,[6] and bile acids[7] by thin-layer chromatography for the detection of natural steroids and for finding new components. In the present work of this series, systematic analysis of conjugated and free bile acids in natural bile was carried out for the purpose of applying this method to chemical analysis in clinics and to comparative biochemical studies. At the same time, semiquantitative determination by rapid and easy method using a densitometer was newly developed. It became possible to show species difference in bile components of various animals from the densitogram, and some new components, hitherto undetected, were found.

I. Separation by Thin-layer Chromatography

Various conditions for concurrent and sharp separation by thin-layer chromatography were examined with taurocholate, taurodeoxycholate, taurochenodeoxycholate, tauroursodeoxycholate, taurolithocholate, glycocholate, glycodeoxycholate, glycochenodeoxycholate as the conjugated bile acids, and cholic acid, deoxycholic acid, chenodeoxycholic acid, lithocholic acid as the free bile acids, and cholesterol, using their pure products.

Examinations were made on developers by various combinations of toluene, benzene, ethyl acetate, amyl alcohol, isoamyl alcohol, butanol, isobutanol, ethanol, methanol, dioxan, phenol, acetic acid, formic acid, and water, and the systems of isoamyl alcohol-acetic acid-water and benzene-acetic acid-water were newly adopted. The former system was found to be especially good in reproducibility and had little tendency to tailing. When the identification was difficult, two-dimensional development with two kinds of solvent systems was carried out. The stationary phase was activated silica gel layer. Concentrated sulfuric acid was selected as the coloring reagent since it gave reliable and sharp coloration, with negligible effect on the trace components other than steroids upon densitometry with transmitted light. This reagent, when heated after spraying, was found to show coloration of yellowish brown to glycine conjugate, dark brown to dark purple to taurine conjugate, brown to free bile acids, and purple to cholesterol. These colored spots were found to show different fluorescence under ultraviolet rays, which aided a more reliable identification. For example, separation of deoxycholic acid and chenodeoxycholic acid is difficult, either as a free acid or as a conjugate, but a difference was found in the color tone of their spots.

Components of natural bile were then examined. There are substances in the bile other than bile acids but their quantity is very minute and they are not likely to interfere in the separation of bile acids. Conditions examined as above were used for attempted separation of animal bile acids by application of bile directly on silica gel layer and a sharp separation was found to be possible. Fresh bile *per se*, or methanol solution of dried bile, without any pretreatment, was spotted on the silica gel layer, and separation and detection of each component were found to be possible. Bile examined were from man, monkey, ox, sheep, dog, pig, bear, cat, rabbit, rat, chicken, Mamushi (*Agkistrodon blumhofii*), Habu (*Trimeresurus flavoviridis*), salmon (*Onchorhynchus keta*), rock trout (*Hexagrammos otakii*), Kawahagi (*Monacanthus cirrhifer*), flathead (*Platycephalus indicus*), sea bream (*Pagrosomus major*), flatfish (*Limanda angustirostris*), eel (*Anguilla japonica*), and carp (*Cyprinus carpio*). Chromatographic pattern of these bile components showed a clear difference while individual difference among the same species of animals, examined with three individuals of each animal, was not found. An example of such chromatograms is shown in Fig. 1.

4) S. Hara, M. Takeuchi : Endocrinol. Japon., **10**, 202 (1963).
5) M. Takeuchi : This Bulletin, **11**, 1183 (1963).
6) K. Takeda, S. Hara, A. Wada, N. Matsumoto : J. Chromatog., **11**, 562 (1963).
7) S. Hara, M. Takeuchi : *Ibid.*, **11**, 565 (1963).

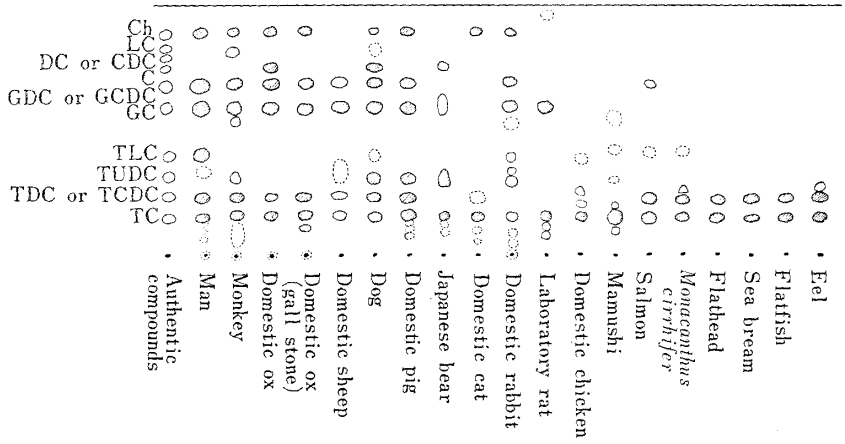

Fig. 1. Chromatogram of Animal Bile

Adsorbent: Wakogel B-5
Developing solvent: iso-AmOH-AcOH-H_2O (18:5:3)
Detection: conc. H_2SO_4, heating

TC: Taurocholate
TCDC: Taurochenodeoxycholate
TLC: Taurolithocholate
GDC: Glycodeoxycholate
C: Cholic acid
CDC: Chenodeoxycholic acid
Ch: Cholesterol
TDC: Taurodeoxycholate
TUDC: Tauroursodeoxycholate
GC: Glycocholate
GCDC: Glycochenodeoxycholate
DC: Deoxycholic acid
LC: Lithocholic acid

II. Densitometry of Chromatograms

In order to obtain a more quantitative data, densitometric examination of colored spots was carried out. As the fundamental experiment, preparation of the plate and conditions for coloring were examined. It was found that the uniformity of the filling density of the adsorbent is more important for obtaining a uniform background state than to make the surface of the thin layer uniformly even.*[6] Uniform spraying of the reagent and uniform heating are necessary for constant tone of coloration. If excess reagent is sprayed or the plate is left in the air for a long time after coloration, the layer will absorb moisture and the amount of transmitted light will change considerably. It is necessary, therefore, to carry out the determination immediately after coloration. Coloration of the developing solvent itself becomes the source of error and removal of the solvent after development must be made thoroughly. It is also necessary to examine whether there would be any difference in the integral value of different compounds in the same amount due to difference in the diffusion of spots from different Rf values or to difference in the intensity and tone of coloration of various compounds. For this reason, thin-layer chromatography was carried out on each pure sample of glycocholate and taurocholate, developing with the same solvent, and the relationship between the amount of sample and the integral of densitogram were examined. It was found that approximate linearity existed between integrated values and quantity of sample in the range of 1 to 30 μg. An example of calibration curve for glycocholate is shown in Fig. 2. The same amount of glycocholate and

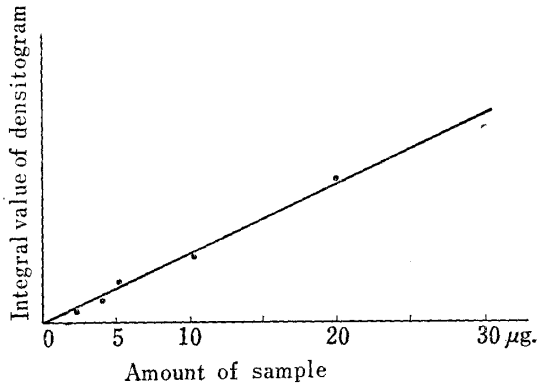

Fig. 2. Calibration Curve for Glycocholate

*[6] Details will be given in the following paper.

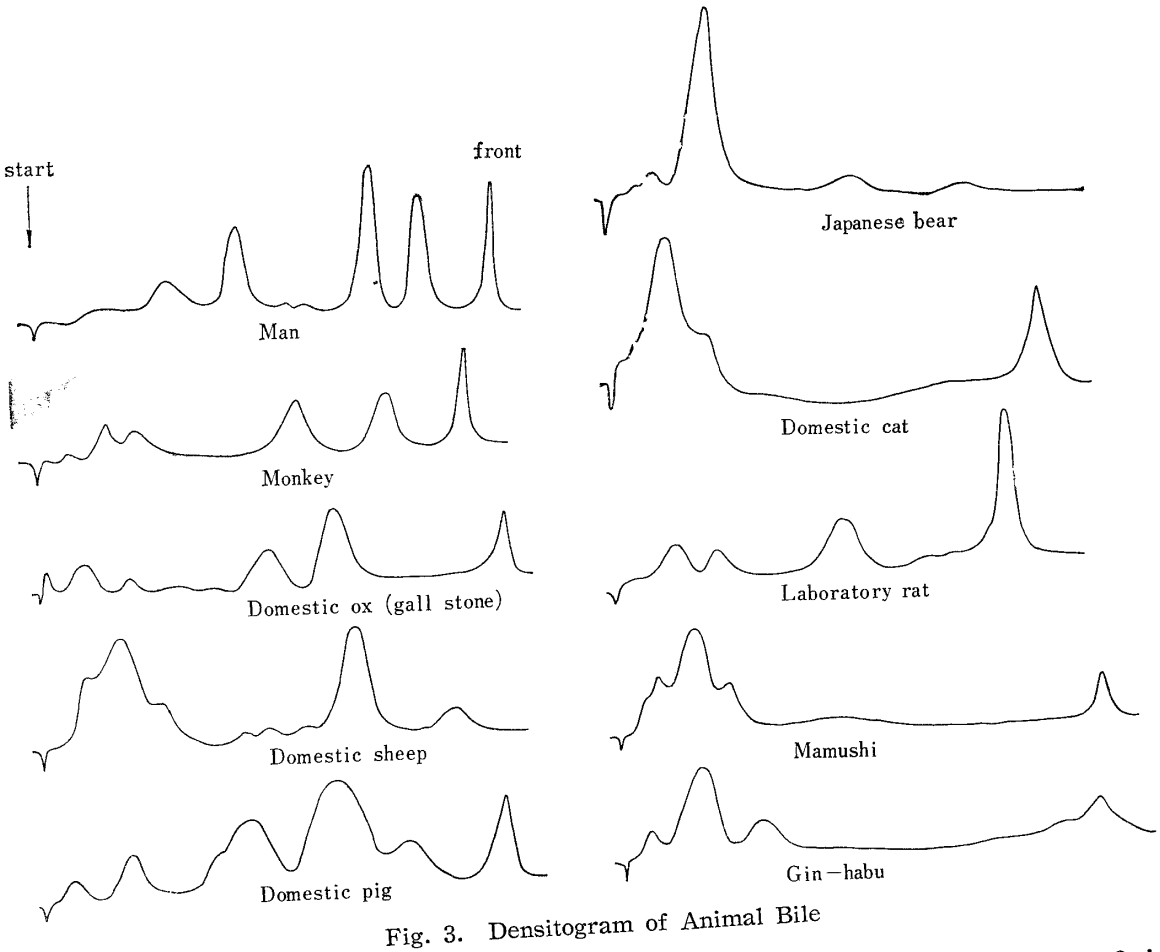

Fig. 3. Densitogram of Animal Bile

taurocholate was found to give approximately the same integral values. Fig. 3 is an example of densitometric examination of chromatograms of bile from various animals carried out under conditions found by above experiments.

The findings in chromatogram, densitogram and color tone are discussed below. It is not possible, however, at the time of writing, to express each of the components in numerical values because of the effect of slight tailing.

Composition of bile is similar in man, monkey, and bovines, the components being taurocholate, taurodeoxycholate or taurochenodeoxycholate, glycocholate, glycodeoxycholate or glycochenodeoxycholate, and cholesterol. A spot was found in the position of taurolithocholate in human bile. Sheep bile showed a spot in the position of taurine conjugate, besides those of glycocholate and glycodeoxycholate or glycochenodeoxycholate. Dog bile was said to have only taurine conjugate, from past observations of infrared analysis, but the bile exhibited spots in the positions of taurocholate, taurodeoxycholate or taurochenodeoxycholate, tauroursodeoxycholate, and taurolithocholate, as well as those of glycocholate, glycodeoxycholate or glycochenodeoxycholate, free cholic acid and cholesterol. Hog bile is known to contain a large variety of cholanic acid derivatives and numerous spots were detected in the positions of taurine and glycine conjugates, and cholesterol. Bear bile contains tauroursodeoxycholate in the majority but minute amount of taurocholate and glycine conjugate seemed to be present, but neither taurine nor glycine seemed to be present in the position of chenodeoxycholate conjugate. Rabbit bile contains glycine conjugate. Rat bile showed the presence of taurocholate, glycocholate and cholesterol but spots were not detected in the position for tauroursodeoxycholate and glycochenodeoxycholate. Chicken bile showed spots for taurocholate and

some spots in the position of taurine conjugates but not of glycine conjugate. Major components in Mamushi and Gin-habu bile appear in the range corresponding to that of taurine conjugates, with Rf values less than 0.2. Fish bile showed not only the spot of taurocholate as reported by many investigators but also some other spots in the position of taurine conjugate. Spots were also found in the position of glycine conjugate in bile of salmon and rock trout. Free acid was practically nonexistent in any of the bile and even if it were present, the amount was extremely minute.

Taurocholate was said to be the main component of carp bile but one of the present authors (G. C.) found that the main component is a sulfate of bile alcohol, since the infrared spectrum of this bile did not show the absorption of carboxylic acid or amide but showed the absorption band of a sulfate, and characteristcs of the spectrum as a whole suggested that the presence of taurocholate as the main component should be denied.[1] Haslewood[8] had obtained a neutral alcohol from the hydrolysate of carp bile but presented no evidence for its structure. Separation of carp bile by silica gel layer, using the afore-mentioned developer, showed one spot in the position different from Rf value of taurocholate and this fact endorsed the result of infrared analysis. It was also found that the sterol obtained by alkaline hydrolysis of the barium salt of this carp bile component gave chiefly four spots by separation through thin-layer chromatography. Comparative examination of the Rf values of synthesized bile alcohols suggested these products to be a triol, tetrol, and pentol.*7 Acetylation of the hydrolysate gave a product which also exhibited mainly four spots. Based on these observations, hydrolysate of the sulfate of the main component was purified by column or thick-plate chromatography.

The foregoing observations support the result obtained from infrared analysis of bile. Further, some minute components, which were difficult to detect by infrared analysis, were also separated and identified. This thin-layer chromatography would be more reliable than infrared analysis for the evaluation of dried bear bile, one of animal drugs. Thin-layer chromatography would also be an effective means for use in clinical examination for continuous, rapid, and simple semiquantitative determination of bile.

Analysis of bile components in cholelithisis and peptic ulcer patients by the present method is now being examined.

Experimental

Preparation of Chromatographic Plate——A thin layer of 250 μ thickness was made on a glass plate, 20×20 cm., with Wakogel B-5 (silica gel for thin-layer chromatography, containing 5% $CaSO_4$ Wako Pure Chemicals, Ltd.) or Kieselgel G (Merck), using the applicator or a spray. Thin layer was also made on a glass plate of 5×20 cm. for insertion into the apparatus for densitometry and uniformity of the layer thickness was preliminarily checked by a densitometer.

Application of the Sample and Development——Taurocholate, taurodeoxycholate, taurochenodeoxycholate, tauroursodeoxycholate, taurolithocholate, glycocholate, glycodeoxycholate, glycochenodeoxycholate, cholic acid, deoxycholic acid, chenodeoxycholic acid, and lithocholic acid were synthesized*8 and each product was confirmed to give only one spot by thin-layer chromatography. These were used as a MeOH solution. Bile was obtained from gall bladder, if present, or from liver bile. Bile itself or MeOH solution of its dried powder, in the amount of 1~10 μl., was spotted at a position 1.5 cm. from one end of the plate, and the spot was dried under an IR lamp.

*7 Thin-layer chromatography of carp bile has recently been carried out by Kazuno and others (J. Biochem. (Tokyo), **54**, 369 (1963)).

*8 Synthesis of conjugate acids followed the method of Bergström and Normann (Acta Chem. Scand., **7**, 1126 (1953); A. Normann: Arkiv Kimi, 8, 331 (1955)).

8) G. A. D. Haslewood: Biochem. J., **59**, xi (1955).

Solvents used for development were each purified by distillation by the usual method. Mixed solvents of iso-AmOH-AcOH-H_2O (18:5:3) and benzene-AcOH-H_2O (10:10:1) were prepared and the plate was developed by the ascending method in a closed vessel saturated with the developing solvent. Lower end of the plate rested in 1 cm. of the solvent. Development of 15 cm. at 20° required about 2 hr. After completion of the development, the plate was heated at 130° for 1 hr. to completely evaporate the solvent.

Colorization and Densitometry——Conc. H_2SO_4 was sprayed uniformly on the developed plate and the plate was heated at 60~80° for 20 min., seeing that the heat was applied uniformly. The plate was submitted to densitometry with transmitted visible light immediately after coloration. The densitometer used was Atago Kogaku Model AG-4 with autorecording integral calculator. Running over a slit for 15 cm. required a few minutes.

Calibration Curves for Glycocholate and Taurocholate——One to 60 μg. of glycocholate or taurocholate was spotted on a uniform plate with constant background and the plate was developed with a solvent system of iso-AmOH-AcOH-H_2O. The plate was colored by the method given above and densitometer was run perpendicular to the direction of development. Absorption curve of transmitted light is calculated automatically.

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Summary

Bile from various animals was submitted directly to thin-layer chromatography, and free and conjugated bile acids and cholesterol in the bile were separated. The chromatogram was analyzed by densitometer with autorecording calculator and animal species difference was revealed by the densitogram. Densitometric analysis gives semi-quantitative results and this can be used for clinical chemical analysis.

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