Acta Medica Okayama

Volume 10, *Issue* 4 1956 *Article* 4

SEPTEMBER 1956

Studies on bile pigments V. A method to isolate indirect bilirubin

Takeshi Sakamoto*

*Okayama University,

Studies on bile pigments V. A method to isolate indirect bilirubin*

Takeshi Sakamoto

Abstract

For the purpose of obtaining the dibasic acid indirect bilirubin in a pure state from the dried canine cholecystic bile, an optimal developing solvent was selected by paper partition chromatography as a preliminary experiment, and it was isolated on cellulose column as an applied experiment. 1. The dibasic acid indirect bilirubin was separable at the starting point in a pure state by paper chromatography under development with the top layer of a n-butanol, acetic acid, water mixture (4:1:5). 2. The dibasic acid indirect bilirubin formed a fixed band at the upper starting place on cellulose column under development with the top layer of a n-butanol, acetic acid, water mixture (4:1:5), and no other substance could be detected there. 3. The dibasic acid indirect bilirubin existing in the fixed band could be eluted out into chloroform with a 1% acetic acid solution. An orange yellow powder was obtained from the eluate by evaporating the solvent in vacuo. 4. Thus separated orange yellow powder agreed well with the crystalline bilirubin in the solubility into organic or inorganic solvents and in the spectrochemical characteristics as well as in the chemical properties.

Acta Med. Okayama 10, 4, 253—260 (1956)

STUDIES ON BILE PIGMENTS

V. A METHOD TO ISOLATE INDIRECT BILIRUBIN

By

Takeshi SAKAMOTO

Department of Internal Medicine, Okayama University Medical School

(*Director : Prof. Dr. K. Yamaoka*)

Received for Publication May 24, 1956.

Several methods have contributed to isolate bilirubin from natural materials[1—7], and the materials availed there were almost gallstones. These procedures lie in an extraction of bilirubin into chloroform from gallstones which were preliminarily hydrolysed with acid and successively washed with alcohol and ether to remove the contaminating impure substances. But it is not a comparatively easy matter to get gallstones of the material in hand, and, on the other hand, there appeared several modifications utilizing bile[8,9] or feces[10,11] as the material. These materials were continuously washed with ether-alcohol in an acid medium, and bilirubin left behind in the residue was extracted into chloroform. A new purifying method of the separated crude bilirubin on alumina column was further reported[12].

There are, on the other hand, different kinds of bilirubin in natural materials in view of their reactivities towards the EHRLICH's diazo reagent, but this fact may well be said to be scarcely mentioned in these reports. The author, therefore, attempted to separate the natural bilirubin according to the attitude towards the EHRLICH's diazo reagent to get some favorable results[13—15]. The present report in this series contains the further surveys on an isolation of the indirect bilirubin.

Experimental

1. *Preparation of Materials.*

Chloroform extract of the dried canine cholecystic bile was

prepared following the procedure of SHIMADA[16]. The crude indirect bilirubin was obtained in an effluate on silica gel column under development with chloroform (Fig. 1), and the crude bilirubin was sampled thereafter after having dried in vacuo.

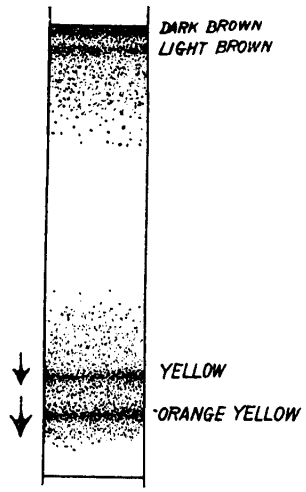


Fig 1. Column chromatogram of chloroform extract of canine bile
Sample: Chloform extract of the dried canine cholecystic bile.
Adsorbent: Silica gel.
Stationary phase: Chloroform.
Developing solvent: Chloroform.

2. *Method.*

An optimal developing solvent for the separating procedure of the indirect bilirubin was preliminarily selected by paper chromatography, and isolation of the pure indirect bilirubin was undertaken on cellulose column under development with the same solvent.

2. 1. *Paper Chromatography.*

The details of the procedure followed that of the preceding report in this series[15].

2. 1. 1. *Samples.*

The crude indirect bilirubin was dissolved in chloroform at the rate of 1 mg/ml, and the crystalline bilirubin was availed for a control, which was also introduced to a chloroform solution in the same concentration as the crude indirect bilirubin.

2. 1. 2. *Filter Papers.*

TOYO No. 51 and SCHLEICHER & SCHÜLL No. 2043 *a* filter papers were availed after no preliminary treatment.

2. 1. 3. *Development.*

After 0.01 ml of the chloroform solution was spotted at the starting point, development was carried out at 12°—14°C by the one-dimensional ascending method.

2. 1. 4. *Detecting Method of Several Substances.*

The GMELIN and EHRLICH's diazo reactions for bilirubin, the SCHLESINGER's reaction for urobilin, the EHRLICH's aldehyde

reaction for urobilinogen, the ammoniacal silver nitrate, aniline hydrogen phthalate, and benzidine reactions for reducing substances, the phosphomolybdic acid, phosphoric acid, and $SbCl_3$ reactions for bile acid, and the ninhydrine reaction for amino acid were availed here.

2. 2. *Column Chromatography.*

2. 2. 1. *Materials.*

The crude bilirubin of 10 mg was dissolved in 2 ml of chloroform.

2. 2. 3. *Columns.*

Five ml of the top layer of a *n*-butanol, acetic acid, water mixture (4 : 1 : 5) was added to 20 g of "Cellulose Powder A" (Toyo Filter Paper Co.), which had been stored in a dessicator, and the cellulose was packed in a Tswett tube of 1.8 cm in diameter after dryness in vacuo. On the other hand, after the sample in a chloroform solution was adsorbed to 1 g of cellulose powder by evaporating the solvent, 0.25 ml of the above top layer was dropped on it and mixed well. Thus treated cellulose was packed further on the column after dryness. One g of cellulose which was treated as in the first was packed furthermore on the column.

2. 2. 3. *Development.*

The same top layer as mentioned above was availed for development, which was carried out in a dark place at 12°—14°C.

2. 2. 4. *Detecting Method of Several Substances.*

Bilirubin was identified by the Gmelin and Ehrlich's diazo reactions, formation of biliverdin by oxidation, detection of mesobilinogen by reduction with sodium amalgam, and recognition of the absorption maximum in the visible range at 450 mμ in a chloroform solution. Fluorescence under the ultraviolet light, the positive Schlesinger's reaction, the negative Ehrich's aldehyde, Ehrlich's diazo, and Gmelin reactions were used for an identification of urobilin, while negativeness of the Gmelin and Schlesinger's reactions and the positive Ehrlich's aldehyde reaction was availed for an identification of urobilinogen. The Salkowsky's reaction for cholesterol and the Casnova's reaction for phosphatide were also availed. Bile acids were detected by $SbCl_3$, phosphomolybdic acid, and phosphoric acid on the paper together with the Pettenkofer reaction in vitro.

Results

1. *Paper Chromatography.*

The crude indirect bilirubin, chromatographed on paper by the top layer of a *n*-butanol, acetic acid, water mixture (4 : 1 : 5), gave a greenish brownish yellow colour near the front together with an orange yellow colour at the starting point (Fig. 2).

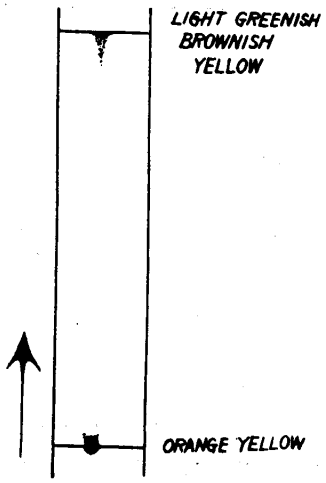


Fig. 2. Paper chromatogram of crude indirect bilirubin

Sample: Crude indirect bilirubin separated from chloroform extract on silica gel column.

Paper: TOYO No. 51.

Development: 30 cm run at 12-14°C by one-dimensional ascending method.

Stationary phase: Water.

Developing solvent: *n*-Butanol, acetic acid, water mixture (4 : 1 : 5)

The former had a greenish yellow fluorescence under the ultraviolet light and the fluorescence was accelerated although it did not change the colour by the SCHLESINGER's reaction, and it gave the positive GMELIN and indirect diazo reactions. The pigment at the starting point grew brown by the benzidine reaction, but it gave neither the ammoniacal silver nitrate reaction nor the ninhydrine reaction. It gave both the positive GMELIN and indirect diazo reactions, but neither fluorescence, the SCHLESINGER's reaction, nor the EHRLICH's aldehyde reaction was given. The phosphoric acid, phosphomolybdic acid, and $SbCl_3$ reactions were all negative there. The pigment was easily dissolved in chloroform, carbon tetrachloride, carbon disulfide, and a $N/10$ NaOH solution. A chloroform solution of the pigment was yellow, and no pigment was transferred into water. The chloroform solution had the absorption maximum at 450 mμ in the visible range and gave the positive indirect diazo reaction, and the hydrochloric azobilirubin had a blue colour. The pigment changed into a green or bluish green colour by the procedures followed that

of LEMBERG and LEGGE[9], and the pigment changed into a substance which gave the positive EHRLICH's aldehyde reaction by reduction with sodium amalgam.

The chromatogram, developed by a mixture or the top layer of a mixture of *n*-butanol, ethanol, water (4 : 1 : 2), *iso*-butanol, acetic acid, water (4 : 1 : 5), *iso*-butanol, ethanol, water (4: 1: 2), or so, had a more or less clear tailing in the frontal region to compare with the above result, and it had also no partitionated substance between the starting point and the front.

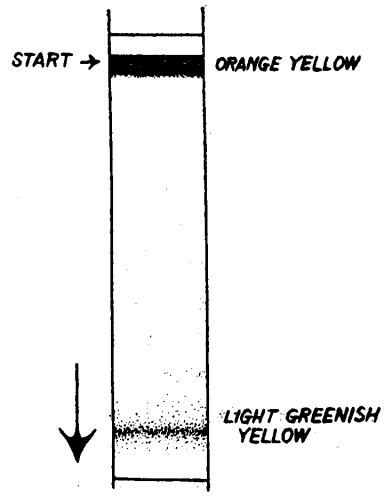
The chromatogram, developed by the top layer of a mixture of *n*-butanol, phenol, water (4 : 1 : 2), *n*-butanol, lutidine, water (4 : 1 : 2), *n*-butanol, collidine, water (4 : 1 : 2), or *n*-butanol, ethanol, ammonia, water (40 : 10 : 1 : 49), showed a remarkable tailing at the starting point and the frontal region.

2. *Column Chromatography.*

The results of column partition chromatography with cellulose powder under development with the top layer of a *n*-butanol, acetic acid, water mixture (4 : 1 : 5) is shown in Fig. 3,

Fig. 3. Column chromatogram of crude indirect bilirubin

Sample: Crude indirect bilirubin.
Column: Cellulose powder.
Stationary phase: Top layer of a *n*-butanol, acetic acid, water mixture (4 : 1 : 5)
Developiug solvent: *do.*



and the crude bilirubin gave two coloured bands, an orange yellow one at the upper starting place and a yellowish green one at the frontal place. The effluate of the latter gave the positive GMELIN and indirect diazo reactions, and the GMELIN reactant grew red in several hours. It gave a light yellow fluorescence under the ultraviolet light, and it was accelerated by the addition of the SCHLESINGER's reagent. It gave a reddish tone very slowly by the addition of the EHRLICH's aldehyde reagent, and the colour turned into green in half a day by let-

ting it standing. The effluate, furthermore, gave neither the positive SALKOWSKY's nor CASNOVA's reaction.

The column was dried as possible after about 250 ml of the solvent passed through it, and then elution of the orange yellow band at the starting place with a 1% acetic acid solution into chloroform gave an orange yellow solution, which was dried in vacuo after washing with water to get an orange yellow powder.

The orange yellow power was well dissolved into chloroform, carbon tetrachloride, carbon disulfide, lutidine, collidine, dioxane, and a $N/10$ NaOH solution, and scarcely into alcohol, but not into water at all. A chloroform solution had the absorption maximum at 450 mμ in the visible range, and gave the positive GMELIN and indirect diazo reactions, but it had no fluorescence under the ultraviolet light, and neither the SCHLESINGER's reaction nor the PETTENKOFER reaction was positive. The oxides of this pigment by LEMBERG and LEGGE's method[9] was green or greenish blue, and the reductants by sodium amalgam gave the positive EHRLICH's aldehyde reaction.

Discussion

When the pigment, which was prepared from the canine cholecystic bile on silica gel column under development with chloroform, was partitionated by paper chromatography with the top layer of a *n*-butanol, acetic acid, water mixture (4: 1: 5), the substance partitionated at the frontal region agreed well with that of the effluate on cellulose column with the same developing solvent. The pigment remained at the starting point in the former also agreed well with the one remained at the starting place in the latter.

That is, both the frontal spot on the paper and the effluate on cellulose column gave the positive GMELIN and indirect diazo reactions, and further both of the two had fluorescences under ultraviolet light and gave the positive SCHLESINGER's reaction. And then, the main substance existing there will be urobilin though the indirect bilirubin may be mingled in part. A brown colour near the front on the paper by the $SbCl_3$ reaction may suggest an existence of a substance like bile acid[10]. To compare

the substance remaining at the starting point on the paper with the one on cellulose column, the former not only gave the positive GMELIN and indirect diazo reactions, but also such reactions as to detect urobilin, urobilinogen, bile acid, steroid, reducing substance exclusive of the benzidine reaction, or amino acid were all negative, and the pigment recognized there agreed well with bilirubin itself together with its transferability into organic or inorganic solvent, and with its absorption maximum in a chloroform solution. And further, the effluate gave the positive indirect diazo reaction, of which colour turned into blue, that of a typical hydrochloric azobilirubin. It was oxidized into biliverdin and reduced into mesobilinogen by sodium amalgam. The above results leave no doubt that the pigment is the dibasic acid indirect bilirubin alone. The latter pigment contained in the upper fixed band on cellulose column could be obtained in an orange yellow powder, which was also identified to the very dibasic acid bilirubin in view of the transferability into organic or inorganic solvent, absorption maximum in a chloroform solution, the positive GMELIN and indirect diazo reactions, transformation into biliverdin by oxidation or into mesobilinogen by reduction with sodium amalgam and so on. Such substances other than bilirubin as urobilin, urobilinogen, bile acid, steroid, reducing substance exclusive of the benzidine reaction, or amino acid were all undetectable. These results also leave no doubt that this band will contain the dibasic acid indirect bilirubin alone as in the case of the starting point on the paper.

On the basis of these data, the most reasonable conclusion may be that this method is very easy and simple for extracting the dibasic acid bilirubin from bile, and that it bears comparison with the methods already reported by many authors in view of an agreement of the result and the theory between a preliminary experiment with paper partition chromatography and an applied experiment with column partition chromatography.

Summary

For the purpose of obtaining the dibasic acid indirect bilirubin in a pure state from the dried canine cholecystic bile, an optimal developing solvent was selected by paper partition chro-

matography as a preliminary experiment, and it was isolated on cellulose column as an applied experiment.

1. The dibasic acid indirect bilirubin was separable at the starting point in a pure state by paper chromatography under development with the top layer of a *n*-butanol, acetic acid, water mixture (4 : 1 : 5).

2. The dibasic acid indirect bilirubin formed a fixed band at the upper starting place on cellulose column under development with the top layer of a *n*-butanol, acetic acid, water mixture (4 : 1 : 5), and no other substance could be detected there.

3. The dibasic acid indirect bilirubin existing in the fixed band could be eluted out into chloroform with a 1% acetic acid solution. An orange yellow powder was obtained from the eluate by evaporating the solvent in vacuo.

4. Thus separated orange yellow powder agreed well with the crystalline bilirubin in the solubility into organic or inorganic solvents and in the spectrochemical characteristics as well as in the chemical properties.

References

1) STÄDELER, F. : Ann., **132**, 323, 1864. — 2) KÜSTER, W.: Z. physiol. Chem., **94**, 136, 1915. — 3) KÜSTER, W. : *ibid.*, **99**, 86, 1917. — 4) KUSTER, W. *ibid.*, **121**, 80, 1922. — 5) KÜSTER, W. and R. HAAS: *ibid.*, **141**, 279, 1924. — 6) FISCHER, H. : *ibid.*, **73**, 204, 1911. — 7) FISCHER, H. and R. HESS : *ibid.*, **194**, 193, 1931. — 8) GIBSON, R. B. and R. C. LOWE: J. Biol. Chem., **123**, xli, 1938. — 9) LEMBERG, R. and J. W. LEGGE: Aust. J. Exptl. Biol. Med. Sci., **18**, 95, 1940. — 10) FISCHER, H. and H. LIBOWITZKY: Z. physiol. Chem., **258**, 255, 1939. — 11) LOWRY, P. T., I. BOSSENMAIER, and C. J. WATSON : J. Biol. Chem., **202**, 305, 1953. — 12) GRAY, C. H. and J. WHIDBORNE : Biochem. J., **40**, 81, 1946. — 13) SAKAMOTO, T. : Acta Med. Okayama, **10**, 11, 1956. — 14) SAKAMOTO, T. : *ibid.*, **10**, 30, 1956. — 15) SAKAMOTO, T. : *ibid.*, **10**, 227, 1956. — 16) SHIMADA, Y. : Igaku Kenkyu, **24**, 1894, 1954 (in Japanese).