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Studies on Bile Pigments I. Separation of Natural Indirect Bilirubins

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Studies on Bile Pigments I. Separation of Natural Indirect Bilirubins*

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Abstract

Descriptions are carried on the method how to separate the indirect bilirubin from the chloroform extracts of the dried dog's gallbeadder bile by adsorption chromatography. 1. The optimal concentrations of the bilirubin content were 2 to 4 mg/100 ml when 1 ml of the sample was adsorbed on the Tswett tube of about 10 mm diameter. 2. Though several zones of the indirect bilirubin were separated on the column of silica gel when developed with various solvents, these zones were proved to be mingled with some oxidized or other intermediate products and the separation like this was thought to owe to the activity of the adsorbents. 3. The chromatogram of the crystalline bilirubin resembled to the one formed by the indirect bilirubin in the chloroform extracts. 4. The chromatogram of the chromatographically separated indirect bilirubin was similar to the former. 5. The absorption maxima of a chloroform solution of the natural indirect bilirubin existed at 450 mμ in the visible range, and it was the same as the maxima of the crystalline bilirubins.

STUDIES ON BILE PIGMENTS

I. SEPARATION OF NATURAL INDIRECT BILIRUBINS.

By

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Introduction

There are two types of bilirubin in serum, that is the so-called direct and indirect bilirubin. The difference between them is based upon the *van den Bergh*'s reaction which is essentially a coupling reaction between the bilirubin and freshly diazotized sulfanilic acid. Though the indirect bilirubin is a normally occurring pigment and couples rapidly with the diazo reagent only when alcohol is added, the direct one couples rapidly and completely with the diazo reagent within half an hour without adding alcohol to produce the same colour as the above. The direct bilirubin appears in serum only when the bile duct system is obstructed and the pigment is thereby reintroduced into the blood stream.

But the essential difference between them is not always concurred among the scholars. It has been believed, for instance, that two types of bilirubin may exist (*Grunenberg*[1,2], *Roberts*[3], *Collinson* and *Fowweather*[4], *Soejima*[5], *Newman*[6], *Griffith* and *Fowweather*[7], etc.), that the difference owes to a combination between bilirubin and protein or other intermediate substances (*Feigl* and *Querner*[8], *Rosenthal* and *Holzer*[9], *Levi-Crailsheim*[10], *Bollman*, *Sheard* and *Mann*[11], *Forrai*[12], *Barron*[13], *Watson*[14], *Cohn*[15], *Najjar* and *Childs*[16], etc.), that the difference does not owe to the molecule of bilirubin (*Thannhauser* and *Anderson*[17], *Weltmann* and *Hueckel* [18], *Heilmeyer* and *Krebs*[19], etc.) especially that the difference owes to the physicochemical characteristics of the sera (*Adler* and *Strauss*[20], *Leschke*[21],

Akiyama[22], *Sofue*[23], *Monasterio*[24], *Acèl* and *Goldgruber*[25], *Takeshita*[26], etc.) and that the difference owes to the bilirubin content and the characteristic of the medium (*Horiuchi*[27], etc.).

As for the important properties to differentiate these direct and indirect bilirubins, *Hoover* and *Blankenhorn*[28], *Leschke*[21], *Brule*, *Garbau* and *Weissmann*[29], *Grunenberg*[1,2] and *Collinson* and *Fowweather*[4] reported the transferability of the pigments into chloroform and the dialysability to collodion membrane; to sum up these reports, the indirect bilirubin is easily transferred into chloroform from the serum and does not pass the collodion membrane by dialysis, but the direct bilirubin, on the other hand, is contrary to the indirect.

In our department *Kosaka*[30,31] divided the direct bilirubin into two forms, the ester-form and the salt-form, by the attitudes towards saponification and hydrolysis. According to *Kosaka*, the ester-form bilirubin transforms into the dibasic acid bilirubin, what is called the indirect bilirubin, by saponification with a 5% methanolic potassic solution, but the salt-form bilirubin transforms similarly into the dibasic acid bilirubin by hydrolysis with a hydrochloric acid solution, and after the incubation of the dibasic acid bilirubin with liver slices it changes into the ester-form direct bilirubin under the influence of liver cells. Therefore, there are three fractions of bilirubin in nature, dibasic acid bilirubins, their esters and salts. The dibasic acid bilirubin is the indirect one and its ester or salt is the direct one. The majority of these direct bilirubins in the human body is of the ester-form, and the salt-form is in a small quantity.

With the progress of the studies in our department it became inadequate to divide the bilirubin into direct and indirect ones, but it may be suitable to do into reactive and inreactive ones towards the diazo reagent. The inreactive bilirubin is the dibasic acid bilirubin, and the reactive one is, as having some reactivities at the propionic acid side chains of the dibasic acid bilirubin, natural ester-form and salt-form bilirubins, bilirubin dimethyl esters, alcoholic molecular compounds and double salts with caffeine as purine base, and other double salts of organic or inorganic acid with the nitrogen of I and IV pyrrolenine nuclei of the bilirubin. When a chloroform solution of the di-

basic acid bilirubin is adsorbed to active alumina, the bilirubin becomes reactive with the diazo reagent. Urobilinogen is also reactive with the diazo reagent, but mesobilirubin is not. In respect to the diazo reaction, on the othe hand, *Fischer* and *Haberland*[32] stated that in the process of this reaction the bilirubin molecule is split by hydrolysis at the central methylene bridge forming vinylneo (isoneo)-xanthobilirubinic acid and that the diazonium salt fastens itself to the free methenyl. *Yamaoka*[33,35], then, believed that reactivity of bilirubin with the diazo reagent was subjected to the activity of central methylene bridge, and that the inreactive bilirubin was the dibasic acid with propionic acid side chains in the form of free acid radicals and the central methylene bridge was stable, but common properties between natural and artificial reactive bilirubins with the diazo reagent are that the oxypyrromethenes on both sides carry some kinds of load and that the central methylene is very susceptible to reaction, and also that when a load is attached to the oxypyrromethenes on both sides of the dibasic acid bilirubin, the central methylene is activated, and becomes reactive with the diazo reagent.

As for fractionation of natural bilirubins it is easy to extract the dibasic acid bilirubin from serum, bile and jaundiced urine with chloroform. According to *Hara*[36] and *Kosaka*[30,31], the salt-form bilirubin is extractable from jaundiced urine by shaking the urine with chloroform, when the salt-form bilirubin is separable as a milky layer between urine and chloroform. As for the ester-form bilirubin, on the other hand, the bilirubin is precipitated from jaundiced urine by saturation of ammonium sulfate, then precipitates are washed with ammonium sulfate solution and dissolved into a 1/10 *N* NaOH solution. After acidifying the solution with hydrochloric acid and repeated extraction of the dibasic acid bilirubin with chloroform, the ester-form bilirubin remains in the mother liquid. *Shimada*[37] separated clearly two fractions of the direct bilirubin from bile on the column of silica gel, when the ester-form bilirubin thus separated differed remarkably in various points from the indirect and the absorption maxima of the separated ester-form bilirubin existed at 425—430mμ in dog's bile and at 430—440mμ in human bile, while the indirect at 450 mμ.

It was then desired to obtain further informations how to separate and isolate the natural bilirubin, indirect and direct and furthermore ester-form and salt-form ones, and as to whether the method of separation affects the character of each bilirubin obtained.

Experimental

1) Materials.

Chiefly dog's gallbladder bile was used, and the crystalline bilirubin was offered for the comparison of separation withthe natural bilirubin.

A) Chloroform Extracts of the Dried Dog's Gallbladder Bile.

Shimada's method[37] was adopted for manufacturing procedures although reformed in part. After preparating gallbladders by ligatures aseptically as possible in operated dogs, they were left in a thermostatic incubator at 37°C for about 4 to 5 days to be dried in a dark brown sand-like or stone-like state. Then to 10 to 15 g of the dried bile was added about 100 ml of chloroform, and kept in an incubator, being frequently shaken for about half a day. Then the bilirubin having been contained in the bile was largely transferred into the chloroform medium. The chloroform medium was then centrifuged for about 30 minutes at 2, 000 r.p.m. when a chloroform phase was gotten in a dark brown to brownish solution. After filtration of the chloroform phase the filtrate was offered as the

Table 1. Changes of the Bilirubin Content in the Chloroform Extracts in Each Elapsed Time. mg/100 ml

Elapsed time.	Indirect bilirubin.	Direct bilirubin.	
		Salt-form.	Ester-form.
Biginning.	2.1	5.3	6.4
After 3 days.	2.2	5.1	6.8
〃 1 week.	2.2	5.4	6.3
〃 2 weeks.	2.3	5.1	5.8
〃 4 〃	2.2	5.2	5.1
〃 8 〃	2.3	5.0	5.2

Bilirubin contents were calculated by *Hara*'s method.

chloroform extracts. According to *Shimada* the chloroform extracts thus separated would contain all the indirect bilirubin and a large quantity of the direct bilirubin. Though bilirubin content of the chloroform extracts differs individually, three fractions of bilirubins were almost constantly extracted, then the chloroform extracts were availed as the sample hereafter.

The chloroform extracts thus prepared were so stable as the ingredients of them suffered scarcely a change for a month or so when stored in a cool and dark place (Table 1.).

B) Chromatographically Separated Indirect Bilirubins.

When development was carried out with a chloroform-methanolic (20 : 3) mixture after adsorption of the chloroform extracts on a column of silica gel, three moving coloured zones were separated. The first zone contained the indirect bilirubin only, and the effluent of this zone was collected and dried in vacuo, and then light reddish brown semicrystalline powders were obtained. They were purified before use as usual — eluting out the upper fixed brown zone into chloroform with a 1% acetic acid aqueous solution after washing the adsorption column of aluminium oxide which was adsorbed beforehand with its chloroform solution, then the chloroform solution was washed with water several times and after drying in vacuo it was dissolved into chloroform again. Thus purified chloroform solution was used as the matrial of the chromatographically separated indirect bilirubin.

C) Crystalline Bilirubin.

Chloroform solution of the crystalline bilirubin supplied from Eastman Kodak Co. was purified similarly with the adsorption column of aluminium oxide. Purified dry bilirubin was introduced before use to a chloroform solution.

II) Methods.

Column chromatography, especially adsorption chromatography, was chiefly availed.

A) Adsorbents and Adsorption Columns.

a) Silica Gel.

Silica gel was supplied from Kanto Chem. Co. under the name of "*Silica Gel for Chromatographic Use*". After activating a proper amout of silica gel by heating it in a china vessel on a sand bath for about 30 min at 130 to 150°C it was cooled in

a thermostatic incubator, and then about two to three volumes of the solvent were added to it as a fixed phase to make the mixture in a semitransparent gel state. The silica gel suspension was thereby poured into a Tswett tube of 30 cm in length and 1 cm in diametre and paked with a cotton plug beforehand, and then in some minutes silica gel would precipitate on the cotton plug and the added solvent would be separated on the column. As soon as the excess of the solvent flowed down the samples were poured on it gently.

Chiefly chloroform and sometimes water, ligroine or benzene were used as the fixed phase.

b) Aluminium Oxide.

"*Aluminium Oxide Standardized* (Brockmann)" or "*Active Alumina* (Takeda)" was used. To some amounts of aluminium oxide activated beforehand by heating as in the case of silica gel at 130 to 150°C for 30 min, an equal to twice the volume of the solvent was added as the fixed phase, and the adsorption column was formed like the above silica gel column.

Chloroform, benzene and water were used as the fixed phase.

B) Development.

As soon as the solvent in the sample poured on the column grew less, developing solvent was adjusted on the surface of the column taking care not to make the surface dry or maintaining the height of the solution to be fairly constant to make it flow down at an equal rate. The development was carried on in a dark place at room temperature (14 to 20° C). No inactive gas was streamed on the surface of the solvent in the glass vessel.

C) Measurement of the Rate of Flow.

In necessity, the rate of flow was expressed conveniently according to *Kuwada*[38] as a ratio of the distance of the moved zone to the distance of the moved meniscus of the developing solvent.

D) Fractionation Test.

When a moved zone was not so clearly recognizable, 2 to 5 ml of the effluent was taken continuously into a test tube to carry out further examinations quantitatively and qualitatively.

E) Optimal Concentrations of Bilirubins in the Sample.

Developments were carried out with chloroform-methanolic (20 : 3) mixtures. The fractions of the effluent were collected from the columns of silica gel, on which the samples in various concentrations were adsorbed beforehand, and then the bilirubin contents in each fraction were calculated according to *Hara*'s method[36].

The optimal separation of the moved zones was seen in the samples of a concentration of 2 to 4 mg/100 ml, when the diametres of the Tswett tubes used here were each 10 mm and the samples were each 1 ml (Table 2.).

Table 2. Bilirubin Contents in Each Effluent. mg/100 ml

Fraction No. (2 ml each)	I	II	III	IV	V	VI	VII
	1.05	2.12	3.20	4.43	5.36	8.18	19.65
1.	—	—	—	—	—	—	—
2.	—	—	—	—	—	—	—
3.	0.08	0.10	0.21	0.28	0.12	0.34	0.82
4.	0.20	0.80	0.93	1.08	0.99	1.92	7.69
5.	0.11	0.10	0.20	0.24	0.14	0.44	2.59
6.	0.08	0.03	0.12	0.14	0.10	0.23	1.90
7.	0.03	—	0.06	0.10	0.14	0.30	1.33
8.	0.03	—	0.04	0.03	0.21	0.41	0.76
9.	0.05	0.04	0.07	0.10	0.10	0.32	0.56
10.	0.04	0.07	0.10	0.14	0.07	0.10	0.38
11.	0.03	0.03	0.11	0.10	0.03	0.10	0.31
12.	—	0.03	0.06	0.03	0.03	0.06	0.24
13.	—	—	0.03	0.03	—	0.03	0.21
14.	—	—	0.03	0.03	—	0.03	0.28
15.	—	—	0.03	0.03	0.03	—	0.17
Column length (cm)	10.8	11.8	13.2	12.0	10.8	10.2	10.6

Fractionation was carried out on each sample on the columns of chloroform packed silica gel, and chloroform-methanol (20 : 3) was used as the developing solvent. Bilirubin contents were calculated by *Hara*'s method. The values on the first line are the initial concentrations of bilirubin in the samples.

Therefore the sample was used in the concentration of 2 to 4 mg/100 ml hereafter.

III) Identification of the Bilirubin.

The identification of both the direct and indirect bilirubins was done in a usual method in our department.

A) Identification of the Indirect Bilirubin.

After evaporating the solvent the pigment was dissolved into chloroform again, and then the indirect bilirubin was easily soluble in chloroform. Although it may dissolve in methanol poorly, it is insoluble in water. When water is added to the chloroform solution of the indirect bilirubin and shaken hard, the bilirubin is not transferred into water. The direct diazo reaction does not occur by adding the diazo reagent to the chloroform solution, but the reaction occurs by addition of alcohol to form the reddish violet azobilirubin which changes into a violet blue hydrochloric azobilirubin when hydrochloric acid is further added. The *Gmelin* reaction occurs, but does neither *Ehrlich*'s aldehyde nor *Schlesinger*'s reaction. The absorption maximum of the chloroform solution exists at 450 mμ in the visible range.

B) Identification of the Direct Bilirubin.

The direct bilirubin has a strong affinity to water and does not dissolve into chloroform. Even when shaken hard after addition of chloroform to it, no pigment in the aqueous solution of it is transferred into chloroform. The direct diazo reaction occurs and a hydrochloric azobilirubin is also formed. Though the *Gmelin* reaction occurs, neither *Ehrlich*'s aldehyde nor *Shlesinger*'s reaction occurs.

In these direct bilirubins, two forms were classified according to hydrolysis or saponification.

a) The Salt-form Bilirubin.

When an aqueous solution of the direct bilirubin was acidified in pH 3.0 or less, some pigments are transferred into chloroform. These pigments have been called the "*salt-form bilirubin*" and the transferability into chloroform has been believed in our department to owe to the changement of the salt-form bilirubin into a dibasic acid bilirubin. But transferability into chloroform does not occur by saponification with a 5% methanolic potassic solution even when the reacted solution is neutralized.

b) The Ester-form Bilirubin.

Except the general characteristics of the direct bilirubin,

chloroform transferability does not appear by acidification with HCl, but does by neutralization after saponification with a 5% methanolic potassic solution.

IV) Quantitative Measurment of Bilirubins.

Quantitative measurements of bilirubin were carried out by *Hara*'s method[36]. To 2 ml of chloroform solution, 6 ml of methanol and 1 ml of the diazo reagent were added and then after about 15 min. 1 ml of conc. hydrochloric acid was further added to put in measurement by *Pulfrich*'s photometer at S_{57} with cuvettes of 10 mm.

Bilirubin content (C) was calculated by measuring the extinction coefficient (E) from the following formula:

$$C = 3.46\ E\ (\text{mg}/100\ \text{ml}).$$

V) Measurements of the Extinction Coefficients.

For the quantitative measurements *Pulfrich*'s photometer (*Zeiss*) was used. But to measure the absorption curves, Electrophotometric Spectrophotometer, Type QB-50 (*Shimadzu*) was used. A tungsten bulb was used as a source of light in the visible range from 350 to 720 mμ, and in the ultraviolet range, on the other hand, a hydrogen discharge lamp was used. Cuvettes were quartz made then.

Results and Discussion

1) Separation of the Indirect Bilirubin from the Chloroform Extracts.

Chromatograms of the chloroform extracts in various developing solvents were classified in the following three types according to the attitudes of the moved coloured zones. The developing solvents of the first type are shown in Table 3, and the moved coloured zones in this type appeared in three places of a, b, and c (Fig. 1.). In the second type only one zone was recognized and Table 4 shows the developing solvents which belonged to this type. But in the third type no moving coloured zone appeared and Table 5 shows the developing solvent.

For chromatographic analysis, the developing solvent which belonged to the second or the third type would not be satisfactory, and so further studies were mainly carried out in the first type. Especially a chloroform-methanolic (20 : 3) mix-

Table 3. Developing Solvents Belonged to the First Type.

1.	Chloroform alone.	
2.	Chloroform-Methanol.	100 : 1 — 5*
3.	Chloroform-Ethyl acetate.	1 : 1/10 — 5**
4.	Chloroform-Amyl acetate.	1 : 1/10 — 50**
5.	Chloroform-Acetone.	1 : 1/30 — 1/5**
		1 : 1/3***
6.	Chloroform-Acetone-Methanol.	100 : 50 : 10 — 50*
7.	Chloroform-Acetone-Ethanol.	100 : 50 : 10 — 50*
8.	Methanol alone. **	
9.	Ethanol alone.	
10.	Diethyl ether alone. *, **	
11.	Diethyl ether-Methanol.	1 : 1/2 — 2
		1 : 3 — 10**
12.	Petroleum ether-Methanol.	1 : 5 — 10
		1 : 12 — 50*, **
13.	Acetone-Amyl acetate.	1 : 2 — 10
		1 : 15 — 50*
14.	Ethyl acetate-Methanol.	1 : 1/10 — 1/2
		1 : 1 — 3**

*..... The c-zone appeared not so clearly.
** ..... The b-zone appeared not so clearly.
***..... The c-zone divided into two zones.

Material ..... Chloroform extracts.
Column..... Chloroform packed silica gel column.

1 ml of the material was availed, and the bilirubin content in the material was 2 to 4 mg/100 ml. Development was carried out on the adsorption column of 10 to 14 cm length.

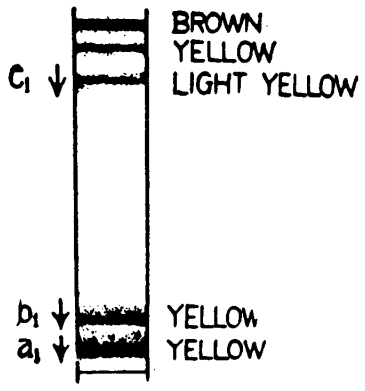

Fig. 1. Chromatogram of the Indirect Bilirubin.
Material·····Chloroform extracts of the dried dog's gallbladder bile.
Column ····· Chloroform packed silica gel column.
Developing Solvent ····· Chloroform-Methanol (20 : 3).

Three moving zones were proved to contain the indirect bilirubin by the indirect diazo reaction and further by the formation of hydrochloric azobilirubin. The direct bilirubin was proved to be fixed at the brown zone after eluting out into water.

ture was used as each moved zone occurred clearly.

Table 4. Developing Solvents Belonged to the Second Type.

1.	Chloroform-Diethyl ether.	1 : 1 — 3
		1 : 4 — 10*
2.	Chloroform-Petroleum ether.	100 : 1 — 100
3.	Chloroform-Ethyl acetate.	1 : 15 — 50*
4.	Chloroform-Acetone.	1 : 10 — 30
5.	Chloroform-Acetone-Methanol.	100 : 50 : 1 — 5
6.	Chloroform-Acetone-Ethanol.	100 : 50 : 1 — 5
7.	Chloroform-Acetone-n-Propanol.	100 : 50 : 1 — 50
8.	Chloroform-Acetone-n-Buthanol.	100 : 50 : 1 — 50
9.	n-Propanol alone.	
10.	n-Buthanol alone.	
11.	Benzene-Methanol.	100 : 1 — 15
		100 : 30 — 50*
12.	Benzene-Diethyl ether.	1 : 1/5 — 1/2
		1 : 1 — 30*
13.	Toluene-Methanol.	100 : 1 — 10
		100 : 15 — 30*
14.	Xylene-Methanol.	100 : 1 — 10
		100 : 15 — 50*
15.	Diethyl ether-Methanol.	1 : 1/50 — 1/2*
16.	Diethyl ether-Ethyl acetate.	1 : 1 — 30
17.	Diethyl ether-Amyl acetate.	1 : 1 — 3*
		1 : 5 — 30
18.	Acetone alone.*	
19.	Acetone-Methanol.	1 : 1/100 — 1*
		1 : 2 — 20
20.	Acetone-Ethyl acetate.	1 : 1/5 — 2*
		1 : 3 — 50
21.	Acetone-Amyl acetate.	1 : 1/5 — 1
22.	Ethyl acetate alone.	
23.	Amyl acetate alone.*	
24.	Petroleum ether-Methanol.	15 — 3 : 1
		2 : 1**
		1 : 1 — 4**, ***

*..... The c-zone appeared clearly, but did not move so remarkably.
** ..... The b-zone appeared, but not so clearly.
***..... The c-zone was divided into two zones.

Materials and adsorption columns were the same as those of Table 3.

Table 5. Developing Solvents Belonged to the Third Type.

1.	Chloroform-Benzene.	1 : 1/100 — 30
2.	Chloroform-Toluene.	1 : 1/100 — 30
3.	Chloroform-Petroleum ether.	1 : 3 or more.
4.	Benzene alone.	
5.	Benzene-Petroleum ether.	1 : 1/3 — 3
6.	Toluene alone.	
7.	Xylene alone.	
8.	Petroleum ether alone.	
9.	Petroleum ether-Methanol.	50 — 20 : 1
10.	Carbon tetrachloride alone.	
11.	Carbon disulfide alone.	
12.	Cyclohexane alone.	

Materials and adsorption columns were the same as those of Table 3.

A) Analysis of the Chromatograms Formed with the Chloroform Extracts.

When developing solvent was applied on the column of silica gel to which chloroform extracts were preliminarily adsorbed, a yellow zone flowed out following the colourless effluent. After the yellow effluent a_1 flowed out, a yellow zone of b did. The effluent b_1 grew faint drop by drop, then c zone flowed out indistinctly. The effluent c_1 of the last zone had a light brownish yellow colour. When the effluents obtained thus were concentrated in vacuo, the colour grew dark brown, light greenish brown, and brownish orange respectively. Being concentrated further, a_1 dried easily, but b_1 and c_1 would not so easily dry. By repeated evaporation and addition of chloroform they dried finally. These pigments were easily transferable into chloroform, but not into water. Mixtures of the diazo reagent and the chloroform solution of these pigments did not show any changes, while after addition of alcohol the typical diazo reaction occurred. Though the indirect bilirubin would surely exist in these pigments, it was suggested that there would mingle some other intermediate substances in them. That is why rechromatography was carried out on the adsorption column of silica gel, ethyl acetate alone beieg used as a developing solvent.

a) Characteristic of the First Effluent a_1.

There were recognized a brown zone at about 5 to 8 mm be-

neath the top and a diffuse light brownish yellow zone between them as fixed zones, but a light brownish yellow zone accompanying with a diffuse light yellow zone moved in the frontal region of the developing. When development was carried on, the frontal coloured zone flowed out. The effluent a_2 could be dried easily in vacuo, and easily dissolved into chloroform but not into water and showed all the properties of the indirect bilirubin. Then the developing solution was changed to methanol, and the brown zone on the surface of the column began to move leaving a gray colour behind. The effluent a_3 was brownish yellow, and after drying the pigment, it could be transferred into chloroform easily, but not into water. This pigment also agreed well with the indirect bilirubin (Fig. 2.).

Absorption maxima of the chloroform solution of the above a_2 and a_3 pigments in the visible range existed at 450 mμ respectively. Though these types of absorption curves have been hitherto believed as those of the so-called bilirubin itself (*Heilmeyer*[39], *Mueller* and *Engel*[40,42], etc.), attention has to be given to the fact that the bilirubin showing these absorption curves would be seen only in the dibasic acid bilirubin as pointed out by *Yamaoka*[43] in 1950 and the so-called direct bilirubin showing the direct diazo reaction would show quite different absorption curves as described below. These facts were corroborated by *Shindoh** who measured the infra-red absorption

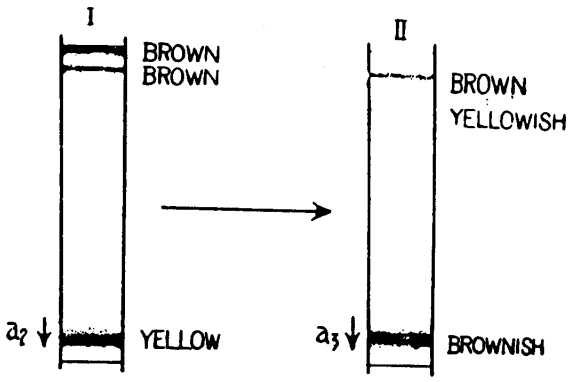

Fig. 2. Rechromatograms of the Effluent a_1.
Material ····· Chloroform solution of the pigment a_1.
Column····· Chloroform packed silica gel column.
Developing Solvents ····· I. Ethyl acetate alone.
II. Methanol alone.
Development was carried out with ethyl acetate alone (I) and followingly with methanol alone (II). The pigments in both a_2 and a_3 were indirect bilirubins, and the absorption maxima existed at 450 mμ respectively in chloroform solutions.

* Personal communication.

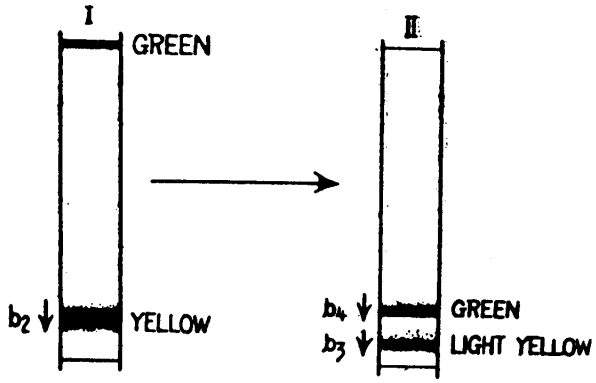

Fig. 3. Rechromatograms of the Effluent b_1.
Material ····· Chloroform solution of the pigment b_1.
Column ····· Chloroform packed silica gel column.
Developing Solvents ····· I. Ethyl acetate alone. II. Methanol alone.
Development was carried out with ethyl acetate alone (I) and followingly with methanol alone (II). The pigments in both b_2 and b_3 were indirect bilirubins, and the absorption maxima existed at 450 mμ respectively in chloroform solutions, but the pigment in b_4 was identified with biliverdin showing the absorption maxima at 640 mμ and 400—390 mμ in methanolic solution.

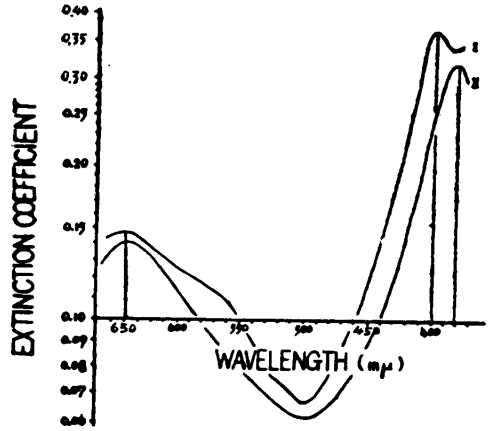

Fig. 4. Absorption Curves of the Effluent b_4.
I ····· Methanolic solution.
II ····· Chloroform solution.
Chloroform solution was prepared as follows: To add some quantities of chloroform and several drops of hydrochloric acid to the methanolic effluent, and then to separate the chloroform phase by addition of a lot of water. The chloroform solution was then washed with water several times and the absorption maxima were measured.
Absorption maxima of the methanolic solution existed at 640 mμ and 400—390 mμ, and minimum at 500 mμ, while those of the chloroform solution existed at 640 mμ and 380 mμ and minimum at 500 mμ.

curves of the dibasic acid bilirubin and its dimethyl ester by *Perkin Elmer 21 C* apparatus. The bilirubin separated or crystallized by *Fischer*[44], *Gray* and *Whidborne*[45] or *Lowry*, *Bossenmeier* and *Watson*[46] and so on, must be, from these view points, also pointed out as the indirect bilirubin, because of their characteritics as well as their extracting procedures being not open to the direct bilirubin.

b) Characteristic of the Second Effluent b_1.

When the material on the column of silica gel was developed with ethyl acetate alone, a yellow zone flowed out (b_2) leaving a

green zone at the top of the column. This flowed-out pigment was similarly identified with the indirect bilirubin after drying in vacuo. Then a green zone accompanying to a yellow zone moved down when developing solvent was changed to methanol alone (Fig. 3.). The yellow flowed-out pigment (b_3) also agreed well with the indirect bilirubin after drying in vacuo. The green pigment (b_4) did not so easily dry as the above yellow pigments without several repeatment of sucking and addition of chloroform. The green pigment was far more easily soluble in methanol than in chloroform. The green pigment in a methanolic solution has the absorption maxima at 640 mμ and 390-400 mμ (Fig. 4.), and then it was identified with the biliverdin.

c) Characteristic of the Third Effluent c_1.

When the chloroform solution of c_1 was developed with ethyl acetate alone similarly, an upper brown zone fixed firmly on the column and did not move easily, though a light yellow zone flowed down faintly (c_2) (Fig. 5.). The flowed-out pigment was also identified with the indirect bilirubin (Fig. 6.).

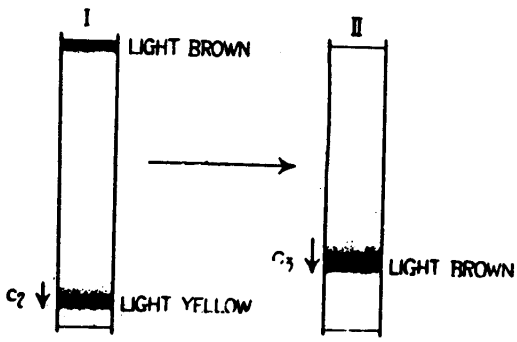

Fig. 5. Rechromatograms of the Effluent c_1.
Material ····· Chloroform solution of the pigment c_1.
Column ····· Chloroform packed silica gel column.
Developing Solvents ··· I. Ethyl acetate alone.
II. Methanol alone.

Development was carried out with ethyl acetate alone (I) and followingly with methanol alone (II). The pigment in c_2 was identified with indirect bilirubin according to the diazo reaction, and the absorption maximum existed at 450 mμ in chloroform solution.

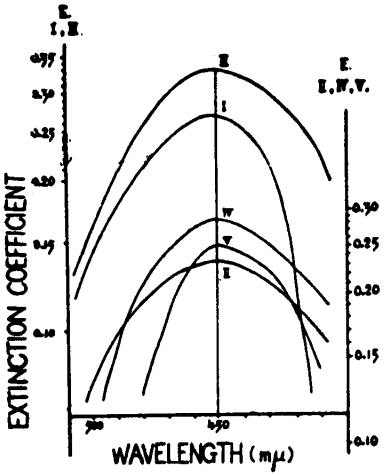

Fig. 6. Absorption Maximum of Each Effluent.

Materials were measured in chloroform solutions, and these maxima agreed well with the indirect bilirubin showing the maxima at 450mμ respectively. I, a_2 ; II, a_3 ; III, b_2 ; IV, b_3 ; V, c_2.

When developing solvent was changed to methanol alone, all the pigments having been left in the upper zone moved down macroscopically, showing a light brownish reddish orange effluent (c_3). This pigment, after drying in vacuo, did not dissolve easily into chloroform but did easily into water and methanol. An aqueous solution of the pigment did not show the diazo reaction, but did slightly the *Gmelin* reaction. When a hydrochloric methanolic solution of this pigment was left alone in a room for a day, the colour grew pale without formation of the green pigment. Judging from these results, this pigment would be a substance changed furthermore than the biliverdin.

B) Attitude of the Direct Bilirubin.

If alcohol content was not so much in the developing solvent, the direct bilirubin was not recognized in the moved coloured zones, while almost all the direct bilirubins were proved in the upper fixed zone by eluting out the pigments into water, but the indirect bilirubin was also recognizable there partly. Though the pigment fixed at 5 to 8 mm beneath the surface was almost the indirect bilirubin, the minority of the direct bilirubin, especially the salt-form, could be seen when the developing solvent contained a large quantity of alcohol or the sample was thick in concentration.

II) Rechromatograms of the Chromatographically Separated Indirect Bilirubin.

To compare the chromatograms of the chromatographically separated indirect bilirubin with the ones formed by the chloroform extracts:

1) In many cases a moved yellow b-zone became faint.

2) A moving yellow c-zone would become unclear.

3) There appeared mostly a slight yellowish colour in the region of the surface.

4) There appeared a yellow fixed zone at 5 to 8 mm beneath the surface as seen in the chloroform extracts, but this zone would become faint if a lot of alcohol was applied to the developing solvent.

After drying the effluent no pigment dissolved into water for all its solubility in chloroform. If water was added to the chloroform solution of the pigments, none of them was trans-

ferred into water after shaking. The pigments fixed in each zone were all well eluted out into an alkaline aqueous solution, where the pigments were transferred easily into alcoholic media by adding some alcohol and ammonium sulfate after neutralization. By evaporating the alcohol after separating from the aqueous media, the pigment was taken in a dry form. This pigment dissolved into chlorform easily, but not into water.

III) Chromatograms of the Crystalline Bilirubin.

The chromatograms of the crystalline bilirubin on the column of silica gel were similar to the above, but the fixed zone at 5 to 8 mm beneath the surface would grow more or less fainter than the former. The pigments, contained in both the fixed and the moved zones, were all the indirect bilirubins and the direct bilirubin was not recognizable.

IV) Studies of the Separated Zones.

The indirect bilirubin, isolated from the first zone (a) on the colum of silica gel by developing with a chloroform-methanolic (20 : 3) mixture from the chloroform extracts, was gathered and stored in dry forms. The pigment was reintroduced into chloroform. The chloroform solution of the indirect bilirubin was subjected again to chromatographic analysis on the column of silica gel by the same developing solvent, when the chromatogram formed was similar to the former. But the chromatogram formed in a shortened length or time by making the column short or sucking with a water jet pump, would not agree to the above, where almost no moved zone was recognized without the frontal zone.

Though these phenomena will be acknowledged as the bilirubin was transformed into some oxidized products during the development in part, separations of several zones will owe to the activity of the adsorbents largely.

Summary

Descriptions are carried on the method how to separate the indirect bilirubin from the chloroform extracts of the dried dog's gallbeadder bile by adsorption chromatography.

1. The optimal concentrations of the bilirubin content

were 2 to 4 mg/100 ml when 1 ml of the sample was adsorbed on the Tswett tube of about 10 mm diameter.

2. Though several zones of the indirect bilirubin were separated on the column of silica gel when developed with various solvents, these zones were proved to be mingled with some oxidized or other intermediate products and the separation like this was thought to owe to the activity of the adsorbents.

3. The chromatogram of the crystalline bilirubin resembled to the one formed by the indirect bilirubin in the chloroform extracts.

4. The chromatogram of the chromatographically separated indirect bilirubin was similar to the former.

5. The absorption maxima of a chloroform solution of the natural indirect bilirubin existed at 450 mμ in the visible range, and it was the same as the maxima of the crystalline bilirubins.

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