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Studies on Bile Pigments II. Seperation of Natural Direct Bilirubins

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Studies on Bile Pigments II. Seperation of Natural Direct Bilirubins*

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Abstract

Separation of both forms of the direct bilirubin were carried out from the dog's gallbladder bile, and further isolations of them were also done. 1. The natural salt-form bilirubin was isolated after separation on the column of aluminium oxide with a n-propanolic aqueous solution. 2. The natural salt-form bilirubin was obtained in amorphous yellow powders which were strongly hygroscopic and easily soluble in water and methanol but not in chloroform or carbon tetrachloride. An aqueous solution of these powders showed both the direct diazo and Gmelin reaction, but neither Ehrlich's aldehyde nor Schlesinger reaction. The salt-form bilirubin was transferred into chloroform only when some quantities of hydrochloric acid were added to a mixture of chloroform and an aqueous solution of it. 3. The absorption maxima of the natural salt-form bilirubin existed at 420 to 430 mμ in a methanolic solution and at 425 or 435mμ in 50% or 10% n-propanol. 4. The natural ester-form bilirubin was isolated after separating on the column of silica gel with a chloroformethanolic mixture. 5. The natural ester-form bilirubin was obtained in amorphous greenish yellow powders. It was further hygroscopic and easily soluble in water and methanol but not in chloroform or carbon tetrachloride. An aqueous solution of it showed the direct diazo and Gmelin reaction, but neither Ehrlich's aldehyde nor Schlesinger's reaction. No pigment was transferred into chloroform even if some quantities of hydrochloric acid were added to a mixture of chloroform and an aqueous solution of it, but did by saponification with 5% methanolic potash. 6. The absorption maxima of the natural ester-form bilirubin existed at 415 mμ in both methanolic and aqueous solutions.

STUDIES ON BILE PIGMENTS
II. SEPARATION OF NATURAL DIRECT BILIRUBINS

By

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Introduction

It has been described in the preceding reports that two types of bilirubin may exist in serum and that these differences have been hitherto based upon the *van den Bergh*'s diazo reaction and further that these two types are different in their physicochemical properties though their chemical structures so far have not been cleared up. But the indirect bilirubin of the dibasic acid bilirubin is separated and isolated from natural materials by several methods, while the direct ones are not isolated owing to their being neglected or to the cause that their basal structures should be the same as the indirect bilirubin though their conjugated substances may be different.

The main purpose of this report existed in isolating these direct bilirubins from natural materials.

Experimental

1) Materials.

Direct bilirubins were prepared as in the following from the chloroform extracts.

A) Methods to Separate the Direct Bilirubin from the Chloroform Extracts.

A dark brown zone moved when development was carried out with water alone on the adsorption column of silica gel which had been preliminarily adsorbed with the chloroform extracts. *Shimada*[1] noted that when silica gel was treated first of all with a buffer solution of pH 7.0 and the same buffer solution was offered as the developing solution, the separation of

the moved zone would become clearer, but the procedures adopted here did not obey to his method because of the defect to mingle the phosphate salt which would complicate the further procedures as described below. The majority of two fractions of the direct bilirubin was recognized in the effluent, but scarcely the indirect bilirubin. Then it was used as the material of the direct bilirubin.

B) Stability of an Aqueous Solution of the Direct Bilirubin.

The colour of this effluent grew remarkably greenish yellow only in a day when it was left standing in a room. This phenomenon was similarly seen even when it was stored in an ice box at 4° to 6°C. The same phenomenon was also seen, though slightly, when toluene was put upon it. A chloroform-ethanolic solution of the direct bilirubin transferred from the above effluent by salting out with ammonium sulfate showed a light greenish yellow colour in two or three days in spite of letting it alone in a room.

These phenomena would mean that the direct bilirubin, especially the one separated thus, would be quite unstable, and the solution was subjected at once to further analysis.

C) Organic Solvent Solutions of the Direct Bilirubin.

Though the bilirubin could be transferred into the chloroform phase partly and in the interphase largely when the effluent was salted out with ammonium sulfate after addition of chloroform, the chloroform phase grew to contain much more bilirubins and the bilirubin in the interphase grew less when ethanol was added to chloroform in half a volume. After collecting the bilirubin in both phases, a small quantity of chloroform-ethanolic (2 : 1) mixture was further added to it and filtrated, and then all the direct bilirubins in the effluent were collected as a chloroform-ethanolic solution, where the direct bilirubin transformed in part into the indirect bilirubin as pointed out by *Shimada*[1].

When a proper amount of ethanol or n-propanol was added to the effluent, the ethanolic or n-propanolic solution of the direct bilirubin could be obtained by salting out in the same way.

In the following experiments the effluent itself was used as an aqueous solution of the direct bilirubin and the organic solvent solution of the direct bilirubin was prepared as above.

These solutions were subjected further to chromatographic analysis.

II) Applications of Adsorption Chromatography.

Silica gel and alminium oxide were availed after activating as described in the preceding report. Adsorption columns were also constructed similarly.

Development was carried out in a usual method in a dark place at room temperature (1°—12°C). The rate of flow was also measured when necessary as described in the preceding report.

III) Identification of the Bilirubin.

The method to identify the indirect and the direct bilirubin, especially both forms of direct bilirubins, the ester-form and salt-form ones, was done in a usual method described in the preceding report.

Results and Discussion

I) Separation of Two Fractions of the Direct Bilirubin.

According to differences of the solvent the description was classified as below.

A) Separation from an Aqueous Solution of the Direct Bilirubin.

a) Separation with Silica Gel.

When water was adopted as a developing solvent, there appeared a brown zone (a) accompanied with a yellow zone (b). The pigments existing in the a-zone were mostly the ester-form bilirubin, although the salt-form would scarcely exist. The pigments existing in the b-zone, on the other hand, were almost the salt-form one and scarcely the ester-form. As a yellow zone remained in the upper region of the column, it would not move in spite of using a large quantity of developing solvent. The pigment contained there was the indirect bilirubin, because the pigment salted out into chloroform after neutralizing the effluent of a 1/10 *N* NaOH solution would not be transferred into an aqueous medium, and because the dried pigment from a chloroform solution thus obtained was also easily soluble in chloroform but not in water (Fig. 1.).

When n-buthanol-saturated water was used as the deve-

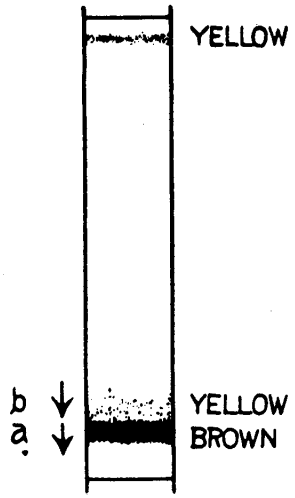


Fig. 1. Chromatogram of the Direct Bilirubin Aqueous Solution on the Adsorption Column of Silica Gel (I).

Material ··· Aqueous solution of the direct bilirubin.

Column ····· Water-saturated chloroform packed silica gel column.

Developing solvent ··· Distilled water alone.

Moving zones were separable in two parts, brown (a) and yellow (b) zones, and a yellow fixed zone was recognizable in the upper region. Bilirubins contained in each zone were:

	Indirect	Salt-form	Ester-form
Fixed yellow zone	(+)	(−)	(−)
Moving yellow b-zone	(−)	(卄)	(∓)
Moving brown a-zone	(−)	(±)	(卅)

loping solvent, a yellow zone appeared in the upper region of the column like the above. It moved quite slowly and the other zones were similar to the above.

When a 10% aqueous solution of n-propanol was used as the developing solvent, two zones, a brown zone at the front and followingly a yellow zone, appeared. The former mostly contained the ester-form bilirubin, although the salt-form would scarcely exist. Though a yellow zone further moved down from the upper region, the pigment existing there was almost the salt-form bilirubin and scarcely the ester-form one. Moreover, two zones were separated from the remaining upper zone, but these zones moved no more (Fig. 2.).

Fig. 2. Chromatogram of the Direct Bilirubin Aqueous Solution on the Adsorption Column of Silica Gel (II).

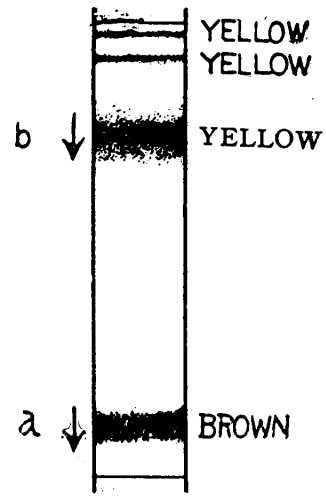
Material ··· Aqueous solntion of the direct bilirubin.

Column ····· Water-saturated chloroform packed silica gel column.

Developing solvent ····· 10% n-propanolic solution.

Moving two zones of a and b were separated leaving two fixed yellow zones behind in the upper region. Bilirubins contained in each zone were:

	Indirect	Salt-form	Ester-form
Fixed upper yellow zone	(+)	(−)	(−)
Fixed lower yellow zone	(+)	(±)	(−)
Moving yellow zone (b)	(−)	(卄)	(±)
Moving brown zone (a)	(−)	(±)	(卅)



b) Separation with Aluminium Oxide.

A yellow moved zone was separated by developing the material with water alone on the column of aluminium oxide. The pigment contained there was mostly the salt-form bilirubin, and scarcely the ester-form one. Moreover, a brown zone was recognized just below the surface. All the pigments in the fixed zone were direct bilirubins when examined after eluting out with a 1/10 *N* NaOH solution. And when development was carried out with a 50% n-propanolic aqueous solution, a clear yellow zone appeared, where the majority was the salt-form bilirubin and the minority the ester-form one. The developing solvent was then changed into a 1/10 *N* NaOH solution, and three moving zones were separated remaining a gray colour at the upper end of the column. The first one of these three zones was yellow and contained mainly the salt-form bilirubin, and the ester-form was scarcely seen. The second zone was brown and contained a large quantity of the ester-form bilirubin and the salt-form bilirubin was very small. The third zone, on the other hand, became obscure and left a light yellowish tone be-

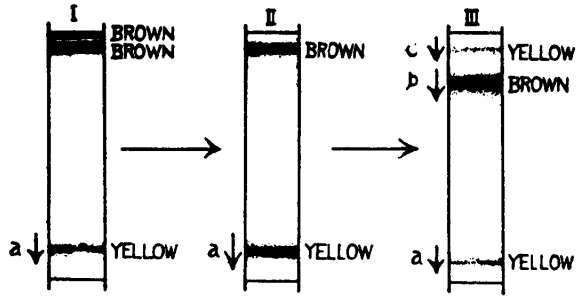


Fig. 3. Chromatogram of the Direct Bilirubin Aqueous Solution on the Adsorption Column of Aluminium Oxide.

Material ··· Aqueous solution of the direct bilirubin.
Column ····· Water packed aluminium oxide column.
Developing solvents: I ····· 10% n-propanolic aqueous solution.
II ····· 50% n-propanolic aqueous solution.
III····· 1/10 *N* NaOH solution.

Development was carried out following the arrow. A yellow c-zone (III) became diffuse and unrecognizable when development was carried on. Bilirubins contained in each moving zone were:

	Indirect	Salt-form	Ester-form
Yellow a-zone of I	(−)	(卄)	(±)
Yellow a-zone of II	(−)	(卄)	(±)
Brown b-zone of III	(−)	(±)	(卅)
Yellow a-zone of III	(−)	(+)	(∓)

hind when the second zone flowed out.

When a 10% n-propanolic aqueous solution was used instead of water as a developing solvent, chromatograms obtained were similar. But the moved zones became clear and the mingled ester-form bilirubin became less. The ester-form bilirubin would not also be seen similarly when developed followingly with a 50% n-propanolic aqueous solution. The chromatogram formed by development with a 1/10 *N* NaOH solution also became similar to the above (Fig. 3.).

c) Separation with a Combination of Silica Gel and Aluminium Oxide.

When a column of silica gel preliminarily adsorbed with an aqueous solution of the direct bilirubin was developed with

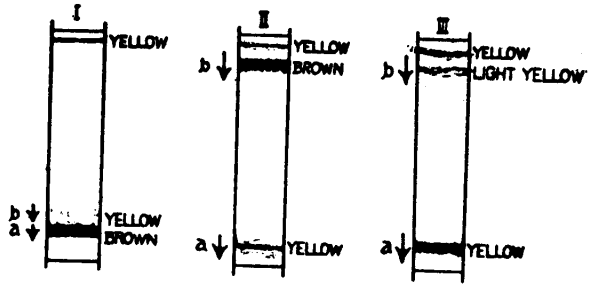


Fig. 4. Separation of the Direct Bilirubin with a Combination of Silica Gel and Aluminium Oxide.

I ··· Chromatogram of the Direct Bilirubin Aqueous Solution on the Adsorption Column of Silica Gel.
Material ····· Aqueous solution of the direct bilirubin.
Column ····· Water-saturated chloroform packed silica gel column.
Developing solvent ····· Distilled water alone.

	Indirect	Salt-form	Ester-form
Yellow b-zone	(−)	(卄)	(∓)
Brown a-zone	(−)	(±)	(卅)

II, III··· Rechromatograms of the Moved Down Direct Bilirubins.
Materials ·····
II···50% n-propanolic solution of the pigment contained in the a-zone.
III···50% n-propanolic solution of the pigment contained in the b-zone.
Columns ····· 50% n-propanol packed aluminium oxide columns
Developing solvents ····· 1/10 *N* NaOH solutions.

A moving yellow a-zone of II became obscure, and a moving light yellow b-zone of III also grew fainter.

	Indirect	Salt-form	Ester-form
Brown b-zone of II	(−)	(∓)	(卅)
Yellow a-zone of II	(−)	(+)	(∓)
Light yellow b-zone of III	(−)	(±)	(±)
Yellow a-zone of III	(−)	(卄)	(∓)

water alone, two zones of a and b were separated like the above. The pigment contained in an a-zone was almost the ester-form bilirubin and the salt-form was quite small. The pigment in a b-zone, on the other hand, was almost the salt-form bilirubin and the ester-form was quite small. After these zones were cut out respectively, the pigment was able to be eluted out each into water. By salting out the eluent with n-propanol and ammonium sulfate, all the pigments were transferred into the n-propanolic phase respectively. Then the n-propanolic phase was separated and furthermore filtrated, and an equal volume of water was applied to the filtrate to be subjected to further analysis. These samples were adsorbed on the column of aluminium oxide packed with a 50% n-propanolic solution, and then developed with 1/10 *N* NaOH solutions. Both forms of the direct bilirubin were more clearly separable than the column of aluminium oxide alone (Fig. 4.).

B) Separation from Organic Solvent Solutions of Direct Bilirubins.

Generally speaking, chromatograms, formed with the materials transferred into an organic solvent itself or an organic solvent containing water from the aqueous solution of the direct bilirubin by salting out with ammonium sulfate, would become more complicated than the one formed directly with an aqueous solution. But it would not matter without the isolation of two fractions of the direct bilirubin. The following methods were not complicated, so they were employed here.

a) Separation of the Direct Bilirubin Transferred into 10% n-Propanolic Solution.

A brown zone, which contained mainly the ester-form bilirubin as well as a small amount of the salt-form bilirubin, was separated in the frontal region when a sample of a 10% n-propanolic solution of the direct bilirubin was developed with a 10% aqueous solution of n-propanol on the column of water-saturated chloroform packed silica gel. A yellow zone moved lately, where existed a large quantity of the salt-form bilirubin containing a small amount of the ester-form. There were two fixed zones in the upper region, yellow and light yellow zones, each of them contained indirect bilirubins but in the latter there was also a small amount of the salt-form bilirubins (Fig. 5.).

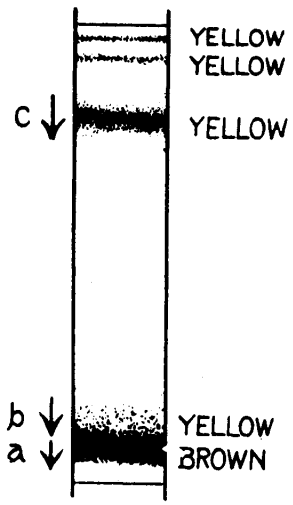


Fig. 5. Chromatogram of a 10% n-Propanolic Solution of the Direct Bilirubin.

Material ····· 10% n-propanolic solution of the direct bilirubin.

Column ····· Water-saturated chloroform packed silica gel column.

Developing solvent ····· 10% n-propanolic solution.

Two moving zones (a and c) were clearly separated, and the frontal zone showed a pretty tailing (b). Two fixed yellow zones were also recognized in the upper region.

	Indirect	Salt-form	Ester-form
Fixed upper yellow zone	(±)	(−)	(−)
Fixed lower yellow zone	(+)	(±)	(−)
Moving yellow c-zone	(−)	(±)	(±)
Tail of the a-zone (b)	(−)	(±)	(+)
Moving brown a-zone	(−)	(±)	(±)

b) Separation of the Direct Bilirubin Transferred into a Chloroform-Ethanolic (2:1) Mixture.

A light yellow zone moved down a little later from the developing front when a sample of chloroform-ethanolic (2 : 1) solution of the direct bilirubin was developed with a chloroform-ethanolic (2 : 1) mixture on the column of ligroine packed silica gel. All the pigments contained in this zone were the indirect bilirubin. Further a blue zone moved a little later, which tied up in their appearances and degrees to the upper green zone, and if eluted out into methanol after cutting out from the column the pigments were recognized as a combination of both the biliverdin and the salt-form bilirubin though the latter was not so much. Bordering the blue zone a yellow or brownish yellow zone appeared clearly. There existed a large quantity of the salt-form bilirubin there. Although a green zone remained in the same place, a yellow zone appeared bordering the green zone and moved down slightly when abundance of developing solvent was supplied. The pigment contained in the yellow zone eluted out easily into methanol after it was cut out from the column, and it was mostly the pure ester-form bilirubin when examined after evaporation of the solvent. The pigment contained in the green zone was recognized as the biliverdin in the similar way (Fig. 6.).

Fig. 6. Chromatogram of the Chloroform-Ethanolic Solution of the Direct Bilirubin.

Material ··· Chloroform-ethanolic (2 : 1) solution of the direct bilirubin.

Column ··· Ligroine packed silica gel column.

Developing solvent ··· Chloroform-ethanolic (2 : 1) mixture.

Three moving zones of a, b and c were separated, and two fixed zones were left in the upper region.

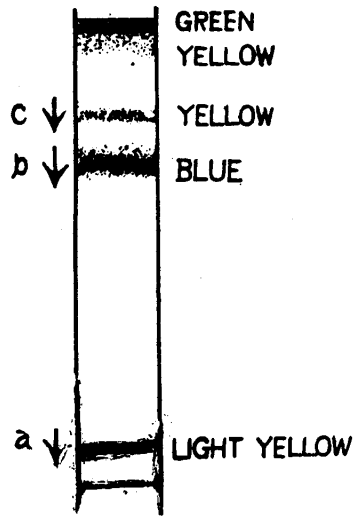
Fixed upper green zone ··· Biliverdin + X.

Fixed upper yellow sone ··· Ester-form bilirubin.

Moving yellow c-zone ····· Salt-form bilirubin.

Moving blue b-zone ····· Biliverdin + X.

Moving light yellow a-zone··· Indirect bilirubin.



II) Isolation of Two Fractions of the Direct Bilirubin.

A) Isolation of the salt-form Bilirubin.

After separation of a yellow zone containing a large amount of the salt-form bilirubin from an aqueous solution of the direct bilirubin on the column of silica gel by development with 10% n-propanol, an aqueous solution of it was adsorbed on the column of aluminium oxide. It was then developed with a 10% and followingly a 50% n-propanolic aqueous solution res-

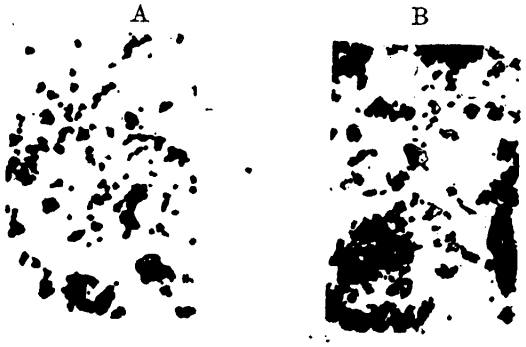


Fig. 7. Microphotograms of the Pulverized Direct Bilirubins.

A ····· A microphotogram of the pulverized salt-form bilirubin.

Isolation of the salt-form bilirubin was carried out with a n-propanolic aqueous solution on the adsorption column of aluminium oxide.

B ····· A microphotogram of the pulverized ester-form bilirubin.

Isolation of the ester-form bilirubin was carried out with a chloroform-ethanolic mixture on the adsorption column of silica gel.

pectively (Fig. 2, cf. Fig. 3.). These solutions were introduced as the materials to isolate the salt-form bilirubin as below.

a) 10% n-Propanolic Aqueous Solutions of the Salt-form Bilirubin.

When furthermore some quantities of n-propanol were applied to the solution, and then the n-propanolic solution was separated by salting out with ammonium sulfate, all the pigments were transferred into the n-propanolic medium. After separation, it was filtrated and dried in vacuo. The pigment was obtained in a spongi-form, which was easily soluble in methanol remaining slightly turbid. Amorphous yellow powders were obtained by drying the filtrate of the methanolic solution.

The powder was easily soluble in methanol and water, and dissolved into carbon disulfide only when a very small amount of methanol existed. But it did not dissolve into chloroform or carbon tetrachloride. It was so hygroscopic as to fuse into an oil-drop-form when it was left standing in a room for several hours. When a mixture of chloroform and an aqueous solution of it was shaken hard, no colour was transferred into chloroform, while all the pigments were transferred into chloroform if some quantities of hydrochloric acid were added to the mixture. By saponification, on the other hand, with a 5% methanolic potassic solution for about 5 min in a boiling-water bath, no pigment could be transferred into chloroform after neutralization. An aqueous solution of it showed the direct diazo and *Gmelin* reaction, but neither *Ehrlich*'s aldehyde nor *Schlesinger*'s reaction. Formation of hydrochloric azobilirubin was typical.

From these results this pigment was identified with the salt-form bilirubin.

b) 50% n-Propanolic Aqueous Solutions of the Salt-form Bilirubin.

After separation and filtration of the n-propanolic phase into which the salt-form bilirubin was transferred, the filtrate was dried and dissolved again into methanol. After filtration and drying of it the residue was washed with chloroform several times, it was dried again, and then yellow amorphous powders were obtained (Fig. 7, A.). The powder was easily soluble in water and methanol, and dissolved into carbon disulfide only

when a very small quantity of methanol existed, but not into chloroform or carbon tetrachloride. It was also quite hygroscopic like the former. No pigment was transferred into chloroform after shaking a mixture of chloroform and an aqueous solution of it, but all the pigments did into chloroform when some amounts of hydrochloric acid were added to the mixture. No pigment was transferred into chloroform by saponification with 5% methanolic potash and following neutralization. An aqueous solution of it showed the direct diazo and *Gmelin* reaction, but neither *Ehrlich*'s aldehyde nor *Schlesinger*'s reaction. Formation of hydrochloric azobilirubin was seen by adding some quantities of hydrochloric acid to the azobilirubin. This pigment was also identified with the salt-form bilirubin.

Two yellow moved zones and a yellow fixed one were seen on the column of silica gel when samples of the carbon disulfide solution of this powder were adsorbed and developed with ethyl acetate alone (Fig. 8.). After developing out the former two zones with ethyl acetate and eluting out the latter into methanol, the properties of these pigments were examined. Although absorption maxima proved respectively existing at 420 mμ, 420 mμ, and 430 mμ, physical properties of them agreed well with the salt-form bilirubin. These pigments may be then somewhat different from one another.

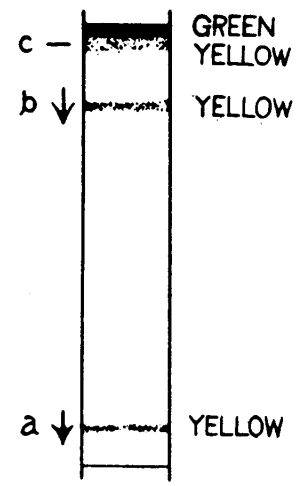


Fig. 8. Chromatogram of the Pulverized Salt-form Bilirubin.
Material···Carbon disulfide solution of the pulverized salt-form bilirubin.
Column ··· Chloroform packed silica gel column.
Developing solvent ····· Ethyl acetate alone.
The pulverized salt-form bilirubin was quite unstable and the yellow colour would turn into greenish in a moist state. This phenomenon was also recognizable on this chromatogram.
Two moving yellow zones as well as a fixed yellow zone were proved to contain the salt-form bilirubin alone. The pigment in the fixed yellow c-zone was eluted out into methanol.
Fixed green zone ····· Biliverdin.
Fixed yellow c-zone ····· Salt-form bilirubin.
Moving yellow b-zone ··· Salt-form bilirubin.
Moving yellow a-zone ··· Salt-form bilirubin.

The absorption maxima of the salt-form bilirubin separated

with a 10% and a 50% n-propanolic solution existed at 425 mμ and 435 mμ respectively (Fig. 9.).

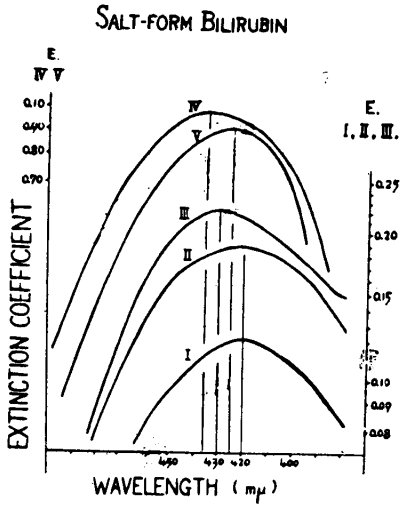


Fig. 9. Absorption Curves of the Salt-form Bilirubin.
I ····· Methanolic solution of the salt-form bilirubin (Fig. 8, a-zone).
II····· Methanolic solution of the salt-form bilirubin (Fig. 8, b-zone).
III ··· Methanolic solution of the salt-form bilirubin (Fig. 8, c-zone).
IV, V ··· 50% (IV) and 10% (V) n-propanolic solutions of the salt-form bilirubins which were separated with 50% or 10% n-propanolic solutions on the adsorption column of aluminium oxide respectively.

Absorption Maxima.

I	420 mμ	IV	425 〃
II	420 〃	V	435 〃
III	430 〃		

B) Isolation of the Ester-form Bilirubin.

Isolating procedures were carried out after the separation of a yellow zone containing a large quantity of the ester-form bilirubin from an aqueous solution of the direct bilirubin on the column of silica gel by developing with 10% n-propanol.

a) Isolation with Aluminium Oxide.

After letting the material adsorbed on a column of aluminium oxide and developing with a 10% and a 50% n-propanolic aqueous solution completely, a 1/10 *N* NaOH solution was availed as a developing solvent, when the first zone of the salt-form bilirubin appeared, though faint, and flowed out in a light yellow colour. But the colour became almost unrecognizable when the second zone of the ester-form bilirubin flowed out (cf. Fig. 4, II.). The effluent of the ester-form bilirubin was light brownish yellow and was an alkaline solution of the almost pure ester-form bilirubin. When salting out was performed with ammonium sulfate on a mixture of this ester-form bilirubin solution preliminarily neutralized by dropwise addition of 1/10 *N* HCl solution and a chloroform-ethanolic (1 : 2) mixture, the bilirubin moved into a lowphase partially and into an interphase largely. After collecting these two phases, and adding some amounts of a chloroform-ethanolic mixture, it became clear, but

the colour turned into greenish yellow and contained ammonium sulfate crystals faintly, although it became quite clear after filtration. It was then dried in vacuo and finally washed with chloroform and ethyl acetate several times and then dried in vacuo completely, and then amorphous greenish yellow powders were obtained. The powder was so hygroscopic as it fuged in an oil-drop-form only in about 20 to 30 min when it was left standing in a room. It was quite soluble in water and methanol, but not chloroform, carbon tetrachloride and carbon disulfide at all. No pigment was tranferred into chloroform when a mixture of chloroform and the aqueous solution of it was shaken hard. This phenomenon was the same when some quantities of concentrated hydrochloric acid was applied to the mixture, but all the pigments were transferred into chloroform when saponification was carried out formally and the solution was further neutralized. An aqueous solution of the pigment showed the direct diazo and *Gmelin* reaction and further formation of hydrochloric azobilirubin, but neither *Ehrlich*'s aldehyde nor *Schlesinger*'s reaction.

When an aqueous solution of it was left standing in a room, a colour of light greenish yellow turned gradually into yellowish green or bluish green in several days. This phenomenon would owe to the lability of this pigment, and the colour of the powders thus obtained would owe to a migration of some oxidized products. For, a green pigment was separated chromatographically from a methanolic solution of this pigment as below and it was identical with the biliverdin.

If development was carried out with ethyl acetate alone on the column of silica gel adsorbed with a methanolic solution of this powder beforehand, two zones of fairly greenish brown and greenish blue were separated. The pigment of the former had all the properties of the ester-form bilirubin without the colour of its fairly greenish tone. Although the latter remained in the upper region, a dark green zone moved down leaving a clear blue zone behind (Fig. 10.). The pigment contained in the moving zone was easily soluble in water and methanol when dried in vacuo, and showed the direct diazo and *Gmelin* reaction, but neither *Ehrlich*'s aldehyde nor *Schlesinger*'s reaction. That is why this pigment would be a mixture of the biliverdin

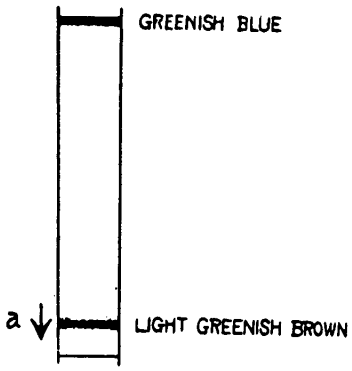


Fig. 10. Chromatogram of the Pulverized Ester-form Bilirubin.
Material ··· Methanolic solution of the pulverized ester-form bilirubin.
Column·····Water-saturated chloroform packed silica gel column.
Developing solvent ····· Ethyl acetate alone.
The effluent had a light greenish brown tone, and contained the ester-form bilirubin showing the absorption maximum at 415 mμ.
The pigment of the fixed zone on the surface of the column was eluted out into methanol, and then it was proved biliverdin.

and the bilirubin. No pigment could be transferred into chloform by shaking the mixture of chloroform and an aqueous solution of this pigment even when concentrated hydrochloric acid was added.

b) Isolation with Silica Gel.

Material was a chloroform-ethanolic (2 : 1) solution of the direct bilirubin prepared by salting out with ammonium sulfate from the aqueous solution of the direct bilirubin. An ester-zone of the column of silica gel developed with a chloroform-ethanolic (2 : 1) mixture (cf. Fig. 6.) was cut out and then the pigment was eluted out into methanol. After drying the methanolic solution, the pigment was washed with chloroform several time, and then dried, and light greenish amorphous powders were got (Fig. 7, B.). A green tone appeared gradually during the purification procedures. The powder was quite hygroscopic, and at the same time so unstable in a moist state that the green tone grew gradually in its degree. It was easily soluble in water and methanol, but not in chloroform, carbon tetrachloride and carbon disulfide at all. Though it would not be transferred into chloroform when a mixture of chloroform and an aqueous solution of it was shaken hard even if some quantities of hyrochloric acid were added, it was easily transferred into chloroform by saponification with 5% metanolic potash. An aqueous solution of it showed the direct diazo and *Gmelin* reaction, but neither *Ehrlich*'s aldehyde nor *Schlesinger*'s reaction. The absorption maxima of a methanolic and an aqueous solution of it existed at 650 mμ, 415 mμ and in the near ultra violet range, and these maxima except the one at 415 mμ would be thought

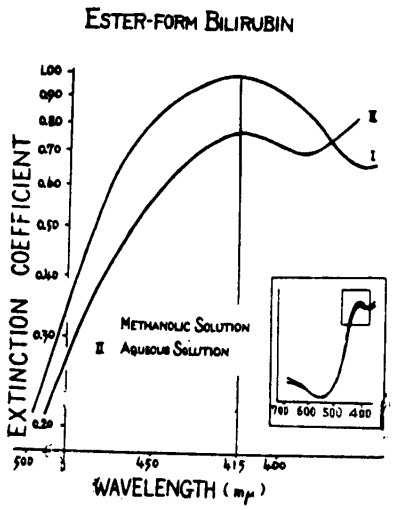


Fig. 11. Absorption Curves of the Pulverized Ester-form Bilirubin.
I ····· Methanolic solution of the ester-form bilirubin.
II ····· Aqueous solution of the ester-form bilirubin.
The ester-form bilirubin was isolated with a chloroform-ethanolic mixture on the adsorption column of silica gel.

to owe to biliverdin derived during the above procedures, and therefore, the proper absorption maximum of this pigment will be recognized as 415 mμ.

These results did not agree with the report of *Kimura*[2] who measured the maxima of chloroform solution of the bilirubin dimethyl ester derived from the crystalline bilirubin, deviating about 10 mμ towards the short wave range. Similarly above results did not agree with the result of *Hosokawa** who measured the maxima of chloroform solution of mesobilirubin dimethyl ester derived from the crystalline bilirubin deviating about 5 to 10 mμ towards the long wave range, and also did not agree with the reports of *Yoshioka*[3] and *Shimada*[1]. But it may be thought that the ester-form bilirubin separated by these methods is more or less differ from the view point of their purities to the one separated by *Shimada* because of his use of phosphate buffer. This was also recognized in point of stability about the case that the ester-form bilirubin isolated by *Shimada* did not change the colour in a short time into green or greenish. These facts will mean that the ester-form bilirubin isolated by these methods is more or less different from the one done by *Shimada*.

* Personal communication.

Summary

Separation of both forms of the direct bilirubin were carried out from the dog's gallbladder bile, and further isolations of them were also done.

1. The natural salt-form bilirubin was isolated after separation on the column of aluminium oxide with a n-propanolic aqueous solution.

2. The natural salt-form bilirubin was obtained in amorphous yellow powders which were strongly hygroscopic and easily soluble in water and methanol but not in chloroform or carbon tetrachloride. An aqueous solution of these powders showed both the direct diazo and *Gmelin* reaction, but neither *Ehrlich*'s aldehyde nor *Schlesinger* reaction. The salt-form bilirubin was transferred into chloroform only when some quantities of hydrochloric acid were added to a mixture of chloroform and an aqueous solution of it.

3. The absorption maxima of the natural salt-form bilirubin existed at 420 to 430 mμ in a methanolic solution and at 425 or 435 mμ in 50% or 10% n-propanol.

4. The natural ester-form bilirubin was isolated after separating on the column of silica gel with a chloroform-ethanolic mixture.

5. The natural ester-form bilirubin was obtained in amorphous greenish yellow powders. It was further hygroscopic and easily soluble in water and methanol but not in chloroform or carbon tetrachloride. An aqueous solution of it showed the direct diazo and *Gmelin* reaction, but neither *Ehrlich*'s aldehyde nor *Schlesinger*'s reaction. No pigment was transferred into chloroform even if some quantities of hydrochloric acid were added to a mixture of chloroform and an aqueous solution of it, but did by saponification with 5% methanolic potash.

6. The absorption maxima of the natural ester-form bilirubin existed at 415 mμ in both methanolic and aqueous solutions.

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[1] *Shimada, Y.*: Igaku-Kenkyu, **24**, 1894, 1954. (in Japanese). — [2] *Kimura, T.*: Igaku-Kenkyu, **23**, 582, 1953. (in Japanese). — [3] *Yoshioka, T.*: Igaku-Kenkyu, **24**, 1422, 1954. (in Japanese).