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Studies on Bile Pigments III. Biliverdins Derived From Natural Bilirubins

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Studies on Bile Pigments III. Biliverdins Derived From Natural Bilirubins*

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Abstract

1. Absorption maxima of hydrochloric biliverdins derived from the natural indirect bilirubin existed at 680 mμ and 375 mμ, but the maxima of biliverdins purified on the column of silica gel existed at 640 mμ and 390 mμ. 2. The natural salt-form bilirubin was oxidized by hydrochloric acid to biliverdin, of which absorption maxima existed at 685 mμ and 370 mμ in a methanolic solution as well as in 5% hydrochloric methanol, but the purified biliverdin in chloroform solution showed the maxima at 640 mμ and 390 mμ. 3. The natural ester-form bilirubin could be transformed into biliverdin by oxidation of its alcoholic solution in the presence of hydrochloric acid. The crude biliverdin had absorption maxima at 645 to 655 mμ, 600 mμ and 320 mμ, and the crude hydrochloric biliverdin had the maxima at 665 to 675 mμ, 620 mμ and in the near ultra-violet range, while the purified biliverdin in chloroform solution had the maxima at 640 mμ and 380 mμ. 4. The biliverdins derived from the indirect, salt-form and ester-form bilirubin had quite similar absorption maxima after purifications by adsorption chromatography.

STUDIES ON BILE PIGMENTS
III. BILIVERDINS DERIVED FROM NATURAL BILIRUBINS

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Introduction

Three fractions of the natural bilirubin, the so-called indirect, salt-form and ester-form bilirubin, were well separable chromatographically on the adsorption column as described in the preceding reports. Though their attitudes towards chloroform transferability, saponification and hydrolysis were quite different, such a colour test peculiar to the bilirubin as the diazo, *Gmelin* and *Fouchet* reaction or a formation of hydrochloric azobilirubin agreed well among them, but their spectrochemical properties were not so. Bilirubin, on the other hand, is apt to transform into biliverdin though poorly in an alkaline solution, which was proved to owe to autoxidation by *Lemberg*[1], while the colour of the isolated salt-form and ester-form direct bilirubins would easily turn into greenish yellow in alcoholic or neutralized aqueous solutions.

The main purpose of this report existed in an attempt to compare the oxidized products among these three isolated natural bilirubins.

Experimental

1) Materials.

Three fractions of the natural bilirubin were isolated and pulverized after separation on the adsorption column from the dog's gallbladder bile as described in the preceding reports. These powders were stored in a dessicator up to use.

A) Oxidation of the Indirect Bilirubin.

a) Hydrochloric Acid Method.

A chloroform solution of the pulverized indirect bilirubin

was added to two volumes of methanol, then concentrated hydrochloric acid was introduced dropwise in a ratio of 5 to 10 vol. % to the mixture, and it was left standing in a dark place at room temperature (10° C), during about 12 to 18 hours a yellow colour of the bilirubin turned into blue to greenish blue, then the chloroform phase was separated by shaking after addition of a large amount of water. The crude biliverdin was obtained in a chloroform solution after washing the chloroform phase with water several times. As the solution might contain pretty amounts of other intermediate substances, it was purified on the adsorption column of silica gel.

b) A Modification of Lemberg and Legge's Method[2].

An equal volume of ethanol containing 1% hydrochloric acid was added to the chloroform solution of the indirect bilirubin, then hydrogen peroxide was added dropwise in 1% with shaking and the mixture was left standing at room temperature (20° to 25° C), and then a yellow colour turned into blue during 15 to 20 min. Further separation and purification of biliverdin followed the above.

B) Oxidation of the Salt-form and Ester-form Bilirubins.

Alcoholic solution of the pulverized salt-form or ester-form bilirubin was added to half a volume of chloroform, then concentrated hydrochloric acid was introduced dropwise in a ratio of 5 to 10 vol. %. The mixture was left standing in a dark place at room temperature (10° C) for about 12 to 18 hours, then a yellow colour of the bilirubin turned into blue to greenish blue. Further procedures were the same as the above.

II) Adsorbents and Chromatography.

Slicia gel was availed after activating as described in the preceding reports. Adsorption columns were formed in a usual method.

Chloroform was chiefly availed as a developing solvent, and the rate of flow was also measured in necessity.

III) Measurements of the Extinction Coefficients.

Electrophotometric Spectrophotometer, Type QB-50 (*Shimadzu*) was used to measure the extinction coefficient, and a tungsten bulb was used in the visible range.

Results and Discussion

I) Biliverdin Derived from the Indirect Bilirubin.

A) Biliverdin Oxidized by Hydrochloric Acid Method.

When concentrated hydrochloric acid was further introduced into a mixture of chloroform-methanolic (1 : 2) solution of the isolated indirect bilirubin, a light brownish yellow solution grew suddenly into light reddish brown. While this solution was left standing in a room for about half a day or more, the colour turned into blue and did not show any yellow tone. Chloroform phase was then separated by adding a large amount of water, and the most of the blue pigments were transferred into chloroform. After washing with water several times the chloroform solution was filtrated, and the filtrate was dried in vacuo. The dried blue pigment was then dissolved into 5 % hydrochloric methanol and the absorption curve was calibrated. The absorption maxima were recognized at 680 mμ and 375 mμ, and the minimum at 490 to 500 mμ (Fig. 1, I.).

The absorption curve was clearly the same as the one of hydrochloric biliverdin and agreed well with hydrochloric biliverdin derived from the so-called bilirubin, the crystalline bilirubin.

When a chloroform solution of the crude biliverdin was adsorbed on the column of silica gel and developed with chloroform alone, a pink zone flowed out first of all, and then a blue zone was clearly separable and was followed by a fairly diffuse violet zone leaving a brown zone behind on the surface of the column (Fig. 2.). The blue pigment in the effluent was introduced into a chloroform solution after evaporating the solvent. When absorption curves were calibrated on the solution, the maxima existed at 640 mμ and 390 mμ and the minimum at 500 mμ (Fig. 1, II.).

B) Biliverdin Oxidized by a Modification of Lemberg and Legge's Method.

When 1% hydrochloric ethanol was added to the chloroform solution of the indirect bilirubin in an equal volume and hydrogen peroxide was further added, the yellow colour turned gradually into blue within 15 to 20 min. The blue pigment was transferred into chloroform by addition of water. The chloro-

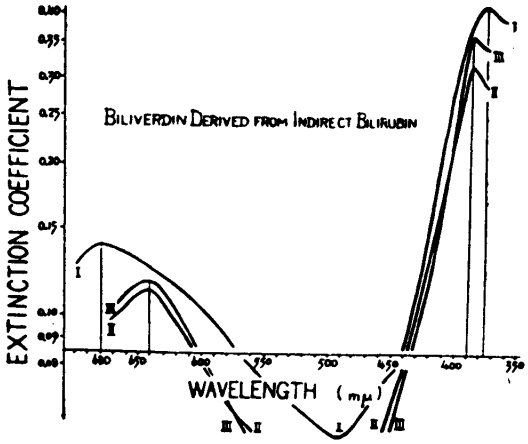

Fig. 1. Absorption Curves of the Biliverdin Derived from the Indirect Bilirubin.

I ····· 5% HCl methanolic solution of the hydrochloric biliverdin, derived from the chloroform-methanolic (1 : 2) solution of the isolated indirect bilirubin in the presence of HCl.

II ····· Chloroform solution of the chromatographically separated biliverdin. The biliverdin was derived from the natural indirect bilirubin in the same way as the above. The crude biliverdin was developed with chloroform on the adsorption column of silica gel, and then purified blue biliverdin was got in powders after drying in vacuo. Chloroform solution of this purified dried biliverdin was subjected to calibration.

III·····Chloroform solution of the chromatographically separated biliverdin. The biliverdin was derived from the natural indirect bilirubin by a modification of *Lemberg* and *Legge*'s method[2]. The crude biliverdin was purified chromatographically like the above, and the purified dried biliverdin was calibrated in a chloroform solution.

	Absorption Maxima.		Absorption Minima.
I	680 mμ	375 mμ	490 to 500 mμ
II	640 〃	390 〃	500 〃
III	390 〃	390 〃	500 〃

form solution was washed furthermore several times with water, and subjected to further analysis.

The chromatogram of this chloroform solution was identical with the former, and a blue zone flowed out lately following a slight pink zone. Absorption maxima of the chloroform solution of the blue pigment after evaporating the solvent of the effluent were recognized at the same place as the former (Fig. 1, III.).

II) Biliverdins Derived from the Salt-form Bilirubin.

A yellow colour of the n-propanolic solution of the salt-

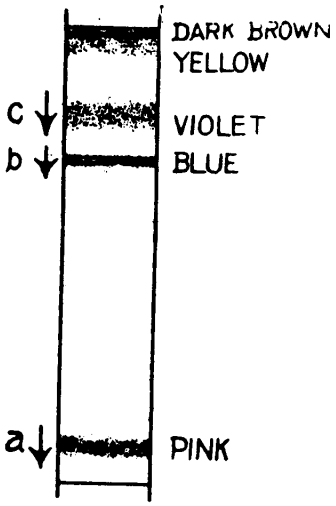

Fig. 2. Chromatogram of the Oxidized Products of the Natural Bilirubin.
Material ····· Chloroform solution of the oxidized products of the natural indirect bilirubin.
Column ····· Chloroform packed silica gel column.
Developing solvent ····· Chloroform alone.
The pigments contained in the b-zone was identified with the biliverdin, showing the absorption maxima at 640 mμ and 390 mμ in chloroform solution, and the chloroform solution of each oxidized products of three fractions of the natural bilirubin formed the same chromatogram.
R (b) = 0.30, R (c) = 0.28.

form bilirubin turned remarkably into blue in half a day or more. The blue pigment was almost completely transferred into a diethyl ether phase when a mixture of the blue n-propanolic solution was shaken hard with some quantities of ether and a large amount of water. After washing the ether phase with water several times, it was dried in vacuo to obtain the blue pigments. The pigment was easily and completely soluble in methanol. The absorption maxima of the methanolic solution existed at 685 mμ and in the near ultra-violet range, and the minimum at 500 mμ (Fig. 3, I.). The absorption curve in 5 % hydrochloric methanol was very similar to the above (Fig. 3, II.). After leaving a 5 % hydrochloric methanolic solution of it standing in a room, it was filtrated and calibrated the absorption curve on the filtrate, and the maxima existed at 685 mμ and 370 mμ(Fig. 3, III.). A yellow colour of a mixture of a methanolic solution of the salt-form bilirubin, chloroform and hydrochloric acid turned into blue in half a day or more. The chloroform phase was then separated, and chloroform was evaporated to obtain the blue pigments. The absorption maxima of the dried blue pigments exsited at 685 mμ and 370 mμ when they were dissolved into methanol (Fig. 3, IV.) or 5 % hydrochloric methanol (Fig. 3, V.).

The dried blue pigment was dissolved into chloroform and was subjected to chromatographic analysis. The chromatogram obtained on the adsorption column of silica gel by development

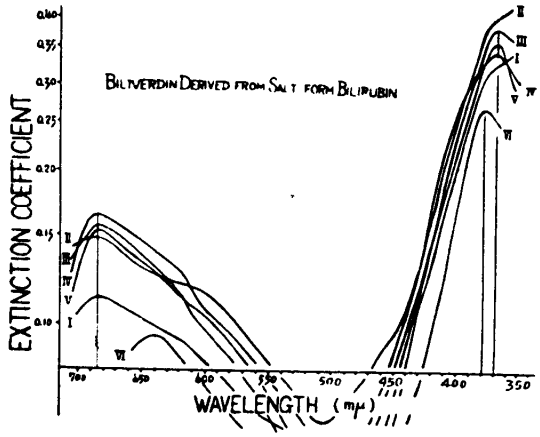

Fig. 3. Absorption Curves of the Biliverdin Derived from the Salt-form Bilirubin.

I·····Methanolic solution of the crude biliverdin which was extracted by diethyl ether from the oxidized products of the natural salt-form bilirubin in n-propanolic solution in the presence of HCl.

II ··· 5% HCl methanolic solution of the crude biliverdin derived like the above.

III···5% HCl methanolic solution of the pulverized salt-form bilirubin, after leaving in a room for a day.

IV··· Methanolic solution of the crude biliverdin which was extracted by chloroform from the oxidized products of the natural salt-form bilirubin in methanolic solutions in the presence of HCl.

V ··· 5% HCl methanolic solution of the crude biliverdin derived like the above (IV).

VI··· Chloroform solution of the chromatographically separated biliverdin. The crude biliverdin was derived like the above (IV). A chloroform solution of the crude biliverdin was developed with chloroform on the adsorption column of silica gel and the purified biliverdin was obtained.

	Absorption Maxima.		Absorption Minima.
I. ·····	685 mμ	near ultra-violet	490—480 mμ
II. ·····	685 〃	near ultra-violet	500—490 〃
III. ···	685 〃	370 mμ	480 〃
IV. ···	685 〃	370 〃	480 〃
V. ·····	685 〃	370 〃	500 〃
VI. ···	640 〃	390 〃	500 〃

with chloroform was quite similar to the former and a blue zone was clearly separated. The absorption maxima of the blue pigment in the chloroform solution existed at 640 mμ and 390 mμ (Fig. 3, VI.) and were identical with the one derived from the indirect bilirubin.

III) Biliverdins Derived from the Ester-form Bilirubin.

A light greenish yellow tone of the methanolic solution of the ester-form bilirubin, isolated with the column of silica gel and further pulverized as described in the preceding report, turned into a clear blue tone in a day when some quantities of concentrated hydrochloric acid had been added beforehand. After separating the blue pigment by addition of chloroform and water like the above, the chloroform phase was washed with water several times and dried in vacuo in a dessicator. The dried blue pigment was then dissolved into methanol (Fig. 4, I.)

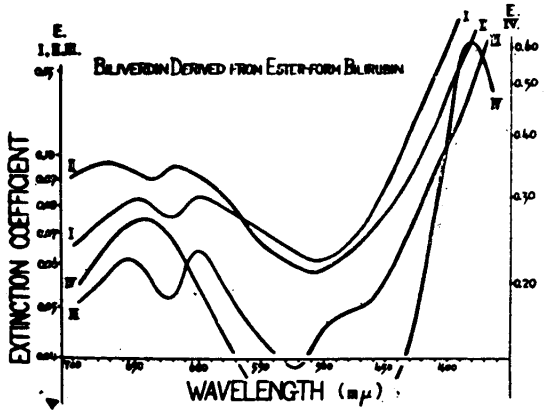

Fig. 4. Absorption Curves of the Biliverdin Derived from the Ester-form Bilirubin.

Biliverdin was derived from the ester-form bilirubin isolated on the adsorption column of silica gel. Oxidation was carried out on a methanolic or an aqueous solution of the bilirubin with HCl, and the blue oxidized products were transferred into chloroform by washing the reactants with water, and then the chloroform solution was dried in vacuo to obtain the green powders.

I·····Methanolic solution of the green powder from the methanolic oxidized products.

II ··· 5% HCl methanolic solution of the green powder from the methanolic oxidized products.

III···Methanolic solution of the green powder from the aqueous oxidized products.

IV···Chloroform solution of the purified biliverdin. A chloroform solution of the green powder from the methanolic oxidized products was developed with chloroform alone on the adsorption column of silica gel to obtain the purified biliverdin of R = 0.30.

	Absorption Maxima.			Absorption Minima.
I·····645 to 655 mμ	600 mμ		320 mμ	510—500 mμ
II ··· 665 to 675 〃	620 〃	near	ultra-violet	510 〃
III···650 to 665 〃	600 〃		320 〃	520 〃
IV··· 640 〃			380 〃	500 〃

or 5% hydrochloric methanol (Fig. 4, II.). The absorption maxima of the former existed at 645 to 655 mμ, 600 mμ and 320 mμ, but of the latter at 655 to 675 mμ, 620 mμ and in the near ultra-violet range, while the absorption minima existed at 510 to 500 mμ respectively. Similarly, after transferring the blue pigment into chloroform from a hydrochloric aqueous solution of biliverdin, chloroform was washed with water several times. After drying in vacuo, absorption curves were calibrated in a methanolic solution (Fig. 4, III.), and the maxima were quite similar to the one (Fig. 4, I.) of the above methanolic solution.

According to *Hosokawa** the absorption maxima at 600 to 620 mμ and in the near ultra-violet range of these curves maybe owe to some further oxidized products. To find a complete explanation of these possiblities, these blue pigments were examined on the column of silica gel by development with chloroform. A pink zone moved down first of all, then a splendid blue zone appeared moving in a low rate of flow (R = 0.30) accompanied with a violet zone in some distance and left a dark brown to blackish zone on the upper surface. The absorption maxima of the chloroform solution of the blue pigment of R = 0.30 existed at 640 mμ and 380 mμ (Fig. 4, IV.).

According to *Lemberg* and *Legge*[3] the absorption maxima of a methanolic solution of the biliverdins existed at 640 mμ and 392 mμ, and of 5% hydrochloric methanol at 680 mμ and 377 mμ. Though these absorption maxima were almost identical with the one derived from the indirect bilirubin as described above, the ones derived from the bilirubins did not agree with these results while they were crude, but the ones purified by chromatography agreed well with those reported by *Lemberg* and *Legge*[3], *Amada*[4] and *Engel*[5]. But when the crude hydrochloric biliverdins derived from the salt-form bilirubin are compared with the one derived from the indirect bilirubin, there is a parallel point in their maxima. These facts may well be explained as follows: When hydrochloric acid is added to the salt-form bilirubin it will be transformed into the dibasic acid bilirubin. Hydrochloric acid will further act secondarily as an oxidizer upon the newly formed indirect bilirubin, and then

* Personal communication.

each hydrochloric biliverdin may resemble closely in their maxima. But the ester-form bilirubin will not be hydrolyzed by hydrochloric acid, and the crude biliverdin derived from it will not agree with the one derived from the indirect bilirubin.

Though it is not easily concluded whether the biliverdins derived from the indirect, salt-form and ester-form bilirubin are identical with or different from one another, the purified ones resembled closely at least.

Summary

1. Absorption maxima of hydrochloric biliverdins derived from the natural indirect bilirubin existed at 680 mμ and 375 mμ, but the maxima of biliverdins purified on the column of silica gel existed at 640 mμ and 390 mμ.

2. The natural salt-form bilirubin was oxidized by hydrochloric acid to biliverdin, of which absorption maxima existed at 685 mμ and 370 mμ in a methanolic solution as well as in 5% hydrochloric methanol, but the purified biliverdin in chloroform solution showed the maxima at 640 mμ and 390 mμ.

3. The natural ester-form bilirubin could be transformed into biliverdin by oxidation of its alcoholic solution in the presence of hydrochloric acid. The crude biliverdin had absorption maxima at 645 to 655 mμ, 600 mμ and 320 mμ, and the crude hydrochloric biliverdin had the maxima at 665 to 675 mμ, 620 mμ and in the near ultra-violet range, while the purified biliverdin in chloroform solution had the maxima at 640 mμ and 380 mμ.

4. The biliverdins derived from the indirect, salt-form and ester-form bilirubin had quite similar absorption maxima after purifications by adsorption chromatography.

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