158. Studies on Bile Pigments. V. Properties of Mesobilirubin

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It may be suggested from the available data achieved in our Department¹⁾ that the aldehyde reagent as well as the Ehrlich's diazo reagent grows reactive on the central methylene radical of bilirubin when something like atoms, atom groups, or molecules combines to both side oxypyrromethenes of the central methylene though bilirubin itself gives no positive diazo reaction.

It was then studied whether mesobilirubin, a reduction product of bilirubin by an introduction of four hydrogen atoms to the bilirubin, may react with the Ehrlich's diazo and aldehyde reagents or not.

Mesobilirubin was prepared after purification of the reactant obtained by the procedure followed that of H. Fischer and G. Niemann.²⁾ The structure of the preparation thus obtained was suggested to be different from that of bilirubin in the state of infra-red absorption of conjugate double bonds at 6.05 to 6.2μ and in disappearance of vinyl radical at 10.09μ , and it was recognized that vinyl radical of bilirubin was undoubtedly reduced to ethyl one. Absorption maxima of the preparation in the visible range lied at $425\text{ m}\mu$ in chloroform and at $426\text{ m}\mu$ in a dioxane solution (Fig. 1), and these data agreed well with those of F. Pruckner and A. Stern.³⁾ The preparation gave positive Gmelin and indirect diazo reactions, but not Ehrlich's aldehyde nor Schlesinger's reaction. The above-mentioned data will undoubtedly show that the preparation is mesobilirubin.

A crystal was then prepared from the mesobilirubin under the action of methanol and hydrochloric gas according to the procedure

Fig. 1. Absorption spectra of bilirubin, mesobilirubin, and dimethyl ester of mesobilirubin
 I...Bilirubin
 II...Mesobilirubin
 III...Dimethyl ester or mesobilirubin

followed that of H. Fischer and G. Niemann.⁴⁾ It was soluble in chloroform and insoluble in an alkali solution contrary to mesobilirubin, and these properties agreed well with those of dimethyl ester of mesobilirubin reported by H. Fischer.⁴⁾ It had an absorption maximum in the visible range at $405\text{ m}\mu$ in chloroform and at $410\text{ m}\mu$ in a dioxane solution (Fig. 1). The shift of the absorption maxima between mesobilirubin and its derivative was well comparable with that between bilirubin at $450\text{ m}\mu$ in chloroform and its dimethyl ester at $425\text{ m}\mu$. These data will show that this derivative is dimethyl ester of mesobilirubin. The dimethyl ester of mesobilirubin was far labile than mesobilirubin, and it gives Gmelin and direct diazo reactions, but not Ehrlich's aldehyde nor Schlesinger's reaction. It is then concluded that a formation of dimethyl ester may be considered as a component of the direct reaction in a chloroform solution on mesobilirubin as in the case of bilirubin.⁵⁾

It was far difficult to make dimethyl ester of mesobilirubin dissolve in serum than to do bilirubin or mesobilirubin, but the dimethyl ester of mesobilirubin dissolved in serum was scarcely transferable into chloroform from the serum solution to compare with the above-mentioned serum solutions of bilirubin and mesobilirubin. These properties may also be comparable with those of dimethyl ester of bilirubin.⁵⁾ Serum solutions of the above three, then, were subjected to hourly spectrophotometric calibrations of direct and indirect reacting bilirubins by the procedure followed that of L. Jendrassik and R. A. Cleghorn.⁶⁾ The volumetric ratio of direct reacting bilirubin to total bilirubin was remarkably high in dimethyl ester of mesobilirubin, and, furthermore, the ratio of the direct reacting of mesobilirubin was so high as in about three times to that of bilirubin (see the table). These results will show that most chemical properties are common between bilirubin and mesobilirubin, and that the latter is apt to grow direct reacting rather than the former.

It will be reasonable to recognize the above-mentioned data to support the prefatory description from the viewpoint that mesobilirubin, a derivative of bilirubin by an introduction of four hydrogen atoms, grew direct reacting towards the diazo reagent though Ehrlich's aldehyde reaction was negative, and that these dimethyl esters were

Fig. 2. Chromatograms of the crude mesobilirubin

Column: Chloroform packed silica gel column
Develop.: Chloroform

Serum solution of bilirubin, mesobilirubin, and dimethyl ester of mesobilirubin

Temp.	Samples	Time	Diazo reaction					Trans-ferability into chloroform
			5 min	10 min	15 min	30 min	60 min	
7°C	Bilirubin	Total	0.121	0.124	0.124	0.124	0.124	+
		Direct	0.000	0.002	0.003	0.010	0.016	
		Ratio (%)					12.90	
	Mesobilirubin	Total	0.152	0.153	0.153	0.153	0.153	+
		Direct	0.002	0.009	0.016	0.039	0.060	
		Ratio (%)					39.21	
	Dimethyl ester of mesobilirubin	Total	0.061	0.061	0.061	0.061	0.061	-
		Direct	0.036	0.051	0.053	0.053	0.053	
		Ratio (%)					86.89	

undoubtedly the direct reacting.

Experimental

1. Preparation of crude mesobilirubin

Mesobilirubin was obtained by the procedure followed that of H. Fischer and G. Niemann.²⁾ The crystalline bilirubin of 30 mg supplied from Eastman Kodak Co. was dissolved into 6 cc of N/10 sodium hydroxide, and the solution was catalytically reduced by 0.3 cc of a 1% colloidal palladium solution. Though it was estimated that

Fig. 3. Hydrogen uptake during mesobilirubin formation from bilirubin

30 mg of bilirubin necessitates 2.6 cc of hydrogen in reduction of bilirubin into mesobilirubin, further addition of palladium was necessary when the reduction grew slow. Hydrogen uptake and hourly progress of the reduction is shown in Fig. 2. Light reddish brownish yellow colour of bilirubin grew hour by hour pale paralleling with the progress of reduction. The resulting solution was arranged to pH 6.5 with a 1% acetic acid solution, and it was repeatedly extracted with chloroform until no colour could be extracted, and then chloroform extraction was once more undertaken at pH 4.0. Thus obtained chloroform extract was washed with distilled water several times, then dehydrated with calcium chloride,

and then chloroform was evaporated in vacuo at 30°C of water bath to get the crude mesobilirubin.

2. Preparation of pure mesobilirubin

The crude mesobilirubin gave a pure mesobilirubin in an effluent of chloroform on silica gel column of 15 cm length supplied from Kanto Chem. Co. (Fig. 3). The yield of thus separated pure mesobilirubin was 10 mg, and it is about 30% to the original sample.

3. Preparation of dimethyl ester of mesobilirubin

According to the H. Fischer and G. Niemann Method,⁴⁾ pure mesobilirubin of 10 mg was suspended in pure methanol of 1 cc, and dry hydrochloric gas was introduced into the suspension under cooling, and then the mesobilirubin grew dissolved but it turned into a yellow plate-like crystal having a greenish red brightness when the hydrochloric gas was saturated.

4. Preparation of serum solutions of bilirubinoid

After the evaporation of the solvent in chloroform solutions of bilirubin, mesobilirubin, and dimethyl ester of mesobilirubin, they had been stored in a vacuum desiccator for 2 to 3 days. These samples were introduced into sound serum of rabbit or dog containing no bilirubin and having no hemolysis, and they were let standing in an ice box for 24 hours. They were then subjected after centrifugation.

5. Calibration of absorption curves

The absorption curves were calibrated with Electrophotometric Spectrophotometer, Type QB-50 (Shimazu) and Recording Spectrophotometer, Model DK (Beckman), and the infra-red absorption spectrum was calibrated with Recording Infra-red Spectrophotometer, Perkin-Elmer 12 C with the kindness of Shionogi Pharmaceutical Institute.

Conclusion

1. Mesobilirubin gave positive indirect diazo and negative Ehrlich's aldehyde reactions in a chloroform solution like bilirubin, but it had a tendency to give a positive direct diazo reaction contrary to bilirubin when dissolved in serum.

2. Dimethyl ester of mesobilirubin gave a positive direct diazo reaction like that of bilirubin.

3. It may be then reasonable to reaffirm our former concept that a combination of atoms, atom groups, or molecules at both side oxypyrrromethenes of the central methylene make the central methylene so active as to be able to react with the diazo reagent.

References

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