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Abstract

Separation of the urinary ester-form bilirubin was attempted, and the results obtained may be summarized as follows: 1. A brown pigment was obtained from jaundiced urine by the following procedures; namely, salting out, methanol extraction, chloroform flocculation, and separation on cellulose column. The pigment has been found to be easily soluble in water, displaying the absorption maximum at 420 - 410 mμ at pH 7.0, and it also gave a positive reaction both to GMELIN's and EHRLICH's diazo reagents within a minute without the addition of alcohol. These characteristics agree well with those of the socalled ester-form bilirubin. 2. On the basis of the results of paper chromatography and paper electrophoresis, the pigment has been determined to contain no amino acid, steroid, nor reducing substance. Moreover, no glucuronic acid could be detected whether examined in vitro or by paper chromatography together with paper electrophoresis, either.

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STUDIES ON BILE PIGMENTS

VIII. A FORM OF DIRECT REACTING BILIRUBINS APPEARING IN JAUNDICED URINE

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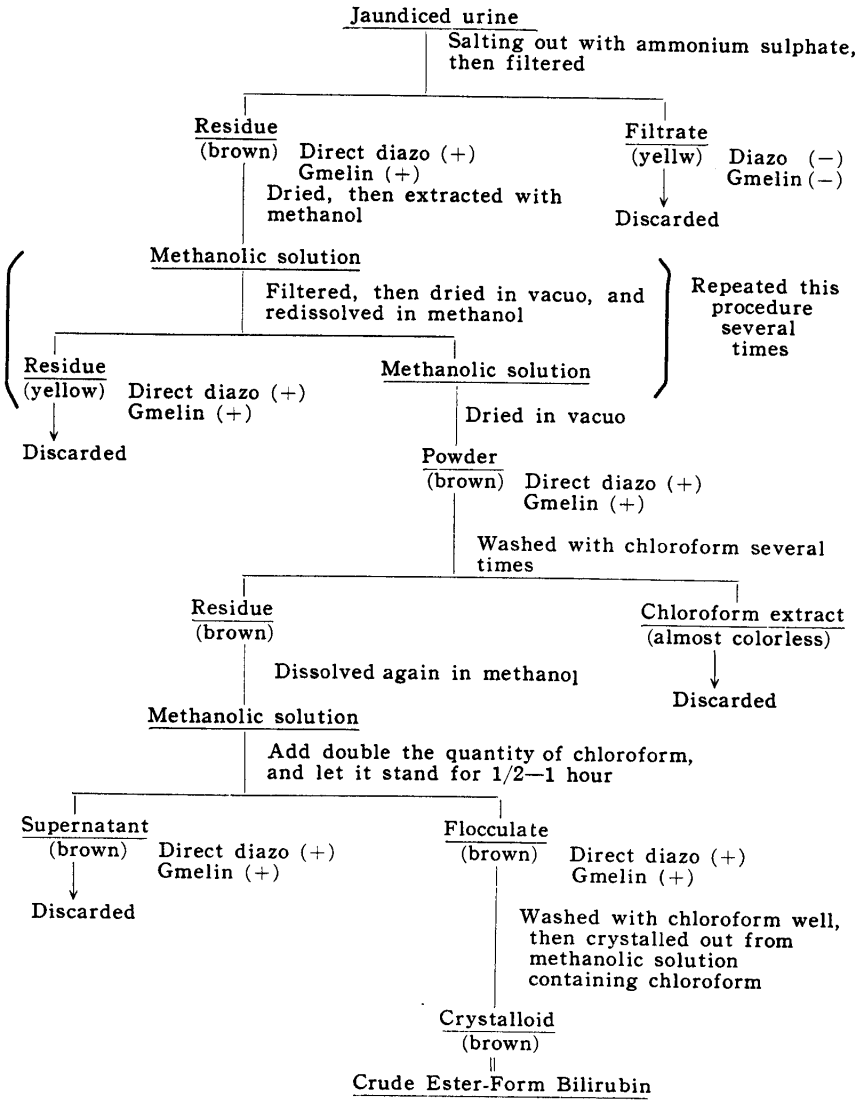
The following is a preliminary report on an investigation being carried out upon the reaction of natural pigments against EHRLICH's diazo reagent, especially, on that of urinary direct reacting pigment appearing in jaundice.

Almost all the pigments giving a positive direct diazo reaction could be separated from diazo-negative urinary pigments by salting out with ammonium sulphate. These pigments were successively extracted with methanol, and brown crystalloid as shown in Table 1, was obtained by further purifying procedures.

This crystalloid has been found to be easily soluble in alcohol and more soluble in water, whereas it is insoluble in chloroform, carbon tetrachloride, benzene, or ether. Its aqueous solution displayed the absorption maximum at 410—400 mμ at pH 7.2, and showed no fluorescence under ultraviolet ray. It was negative to both EHRLICH's aldehyde and SCHLESINGER's reactions, but without addition of alcohol acted positively to EHRLICH's diazo reaction within one minute, showing a reddish violet color, and likewise positively the Gmelin test. It is well known that dibasic acid indirect bilirubin is soluble in chloroform and insoluble in water or alcohol, and that the chloroform solution displays the absorption maximum at 450 mμ and does not act positively on EHRLICH's diazo reagent without addition of alcohol. In view of these, it may be said that the pigment thus obtained, still possessing the chracteristics of bilirubin as it does, is clearly different from the dibasic acid bilirubin, judging from their positive reactions to both GMELIN and EHRLICH's diazo reagents.

Table 1. Operation to separate a form of direct reacting bilirubins from jaundiced urine



Even after thoroughly mixing this aqueous solution with an equal volume of N/10 hydrochloric acid in an attempt to extract pigment with chloroform, it has been found that no pigment is tranferred to chloroform, but that almost all the pigments are transferred to chloroform after

KOSAKA's saponification procedure.[2] The chloroform solution so obtained shows no fluorescence under ultraviolet ray and acts negatively to EHRLICH's aldehyde or SCHLESINGER's reactions, while presenting a positive GMELIN reaction as well as a positive indirect reaction to diazo reagent. These data seem to suggest that the direct bilirubin possesses all the characteristics of the so-called ester-form bilirubin.[1,2]

On examining this aqueous solution by paper chromatography with the spray of EHRLICH's diazo reagent, no spot such as *o*-aminophenol-, naphthoresorcin-, antimony trichloride-, phosphoric acid-, or phosphomolybdic acid-positive spot could be detected on the chromatographic paper other than a yellow bilirubin spot and amino acid spot with different Rf value, presenting a reddish violet direct diazo reaction within a minute. At this phase of the experiment, various developing solvents and aspects of time elements involved in the preliminary treatment of the paper with the solvent were studied, and as the result, the supernatant of *n*-butanol-ethanol-water in the proportion of 4 : 1 : 2 has been found to be most suitable as the developing solvent for this purpose. However, the question whether or not the pure ester-form bilirubin can successfully be separated at this stage is of interest inasmuch as it depends on whether or not amino acid is eradicatable from the crude ester-form bilirubin. For the purpose of ascertaining this point in question, the crude ester-form bilirubin had been developed on a cellulose column by using the aforementioned supernatant of *n*-butanol-ethanol-water ; and succeeded in chromatographic separation of a single color, yellow pigment as shown in Fig. 1.

This yellow pigment was found to possess all the characteristics of the crude ester-form bilirubin, namely, readily soluble in methanol, and still more soluble in water ; but a paper chromatography revealed that its aqueous solution had the absorption maximum at 420—410 mμ at pH 7.0 and that it was free of amino acid. From these results, it may be said that the ester-form bilirubin so separated on the cellulose column is a direct bilirubin and that it contains lesser impurity than any bilirubins separated from jaundiced urine by other methods.

Recently it has been reported that the direct reacting bilirubin is the one consisting of bilirubin glucuronide[3,4,5] as its principal component, and C. J. WATSON, agreeing to this opinion, has informed in a personal communication to one of the present authors, SAKAMOTO, that "Thus, the former concept that the prompt direct birubin was sodium bilirubinate as advanced by HUNTER and others and that the indirect bilirubin was bilirubin-globin as proposed by DUESBERG, also by POLONOVSKI and FIESSINGER, both of these concepts having been favored earlier by me, must now be abandoned."[6] Further experiments are now being carried out, but from

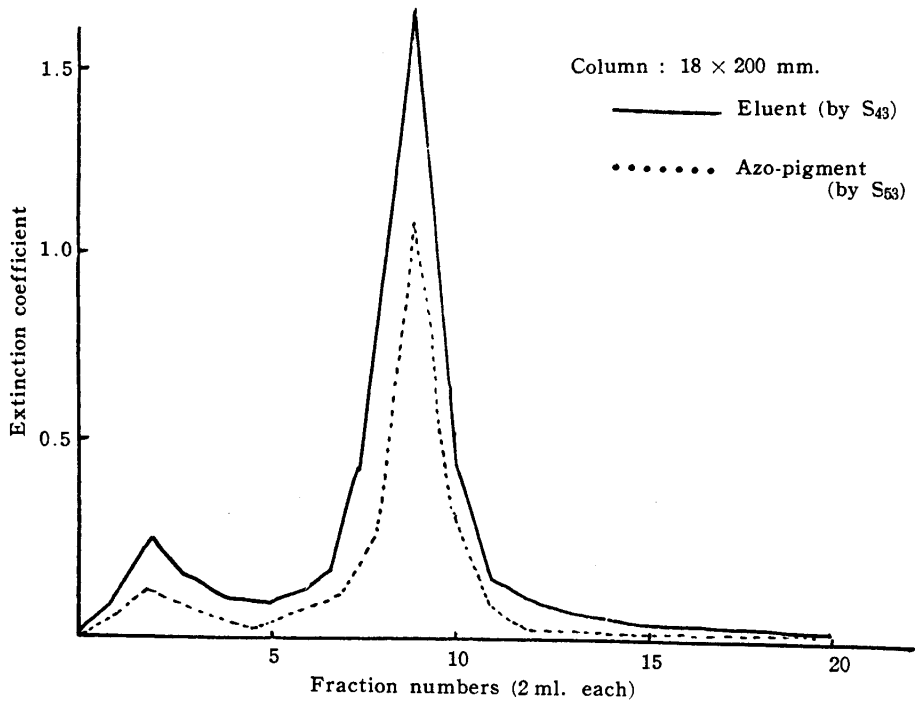


Fig. 1. Elution curve of the crude ester-form bilirubin on cellulose column

the results obtained so far, no evidence has been found to prove that the direct reaction of the ester-form bilirubin is dependent upon a combination of the bilirubin with glucuronic acid. Moreover, since glucuronic acid does not always present itself in the direct reaction of other form bilirubin, it requires further studies to solve the question whether or not bilirubin gluconide is essential in the direct reaction.[7] The definitive explanation on the rôle of glucuronic acid in the direct reaction of bilirubin, however, must await the results of more comprehensible studies on the point that a large quantity of direct bilirubin, other than the bilirubin separated by the present method, still remains in the direct bilirubin extracted from jaundiced urine. On the other hand, when the crude ester-form bilirubin had been examined by paper electrophoresis (Fig. 2), two bands presenting direct reaction were obtained. Of the two, the less stable pigment was readily dissolved in water and its aqueous solution had been found to have the absorption maximum at 420—410 mμ at pH 7.0. And reacting within one minute with diazo reagent without adding alcohol, this solution turned reddish violet, but this pigment proved to be different from the pigment possessing the absorption maximum at 565 mμ, characteristic of naphtho-

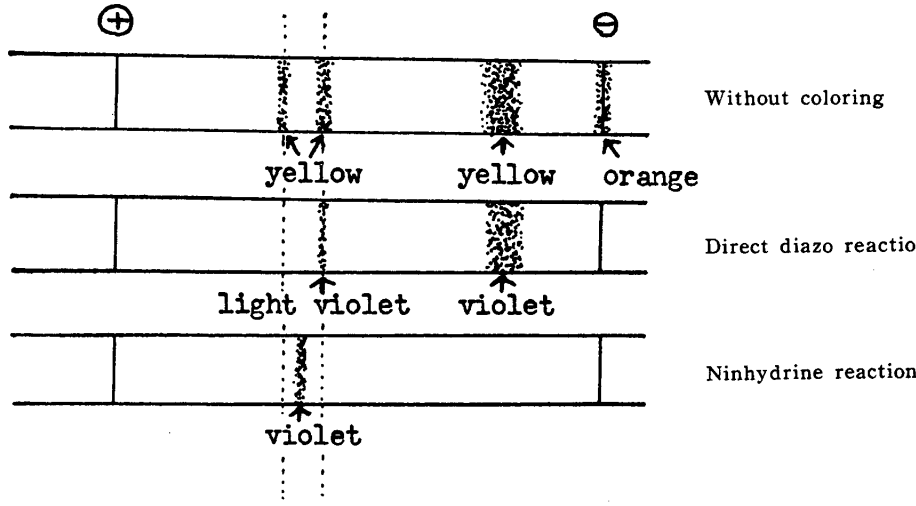


Fig. 2. Paper electrophorograms of the crude ester-form bilirubin

rescin reaction by the ISHIDATE and NAMBARA method.[9]

EXPERIMENT

1. Material. Jaundiced urine from the respective patient described in Table 2 was used. Administration of glucuronic acid preparate was discontinued at least a week before taking their urine.

Table 2. Jaundiced urine employed (in mg./dl.)

No.	Name	Sex	Years	Date	Bilirubin		Urinary urobilinogen (volume)	Diagnosis
					Serum	Urinary		
					Total (Direct)	Total (I. D.)		
1	Y. K.	♂	53	15/Ⅷ (1956)	37.99 (20.00)	6.64 (1.15)	Aldehyde (−) (750 ml.)	Cancer of the amp. of Vater
2	T. N.	♂	49	8/X (1956)	22.75 (15.91)	11.10 (1.18)	0.09 (1,100 ml.)	Chronic hepatitis
3	J. H.	♂	54	9/XI (1956)	20.15 (15.15)	15.74 (3.25)	Aldehyde (−) (1,050 ml.)	Mechanical jaundice
4	K. Y.	♀	49	27/XI (1956)	15.34 (11.16)	9.17 (2.21)	0.07 (800 ml.)	Cancer of bile duct
5	T. S.	♀	41	25/XII (1956)	15.10 (12.10)	6.57 (0.24)	Aldehyde (−) (600 ml.)	Mechanical jaundice
6	T. U.	♂	48	8/Ⅱ (1957)	4.46 (4.08)	1.14 (0.05)	0.18 (1,200 ml.)	Gallstone
7	K. I.	♂	22	13/Ⅱ (1957)	5.60 (5.24)	2.04 (0.28)	2.24 (1,700 ml.)	Acute hepatitis

Abb.: Direct: Direct reacting bilirubin
I. D.: Indirect reacting bilirubin

2. EHRLICH's diazo reaction. The solutions used for EHRLICH's diazo reaction were divided into two groups, namely, solution I which was prepared by dissolving 1 g. of sulfanilic acid and 15 ml. of concentrated hydrochloric acid in 1,000 ml. of distilled water, and the solution II prepared by dissolving 0.5 g. of sodium nitrite in 100 ml. of distilled water. By mixing 10 ml. of the solution I and 0.3 ml. of the solution II before the use, the solution so mixed was used within 5 minutes.

3. Detection of glucuronic acid. For the determination of glucuronic acid *in vitro*, barium naphthoresorcin carbonate method[8] and naphthoresorcin picrate method[9] were employed and an appearance of the absorption maximum at 565 mμ was then considered to be specific to the products of these reactions while on filter paper, *o*-aminophenol method[10] and the ordinary naphthoresorcin method[11] were used for the detection.

4. Paper chromatography. Paper chromatography was carried out by one dimensional ascending method as described in the preceding report.[12]

5. Column chromatography. Column chromatography has been conducted by the method, the details of which are described in the preceding report.[13] Two ml. of each effluent was collected with fraction collector, and after evaporating the solvent under a low pressure the residual pigment was dissolved in 2 ml. of methanol and its extinction coefficient was determined with the filter S_{43}. Then adding 3 ml. of the diazo mixture to this solution the extinction coefficient of the resulting azo pigment was determined with the filter S_{53} 30 minutes afterwards.

6. Paper electrophoresis. The sample was manipulated under the following conditions ; using a paper electrophoresis apparatus, Model KOBAYASI (NATSUME Co.), veronal buffer solution, pH 8.5, 180 V/20 cm., 12 mA/cm. on SCHLEICHER u. SCHÜLL Nr. 2043*a* paper, for 5 hours.

7. Calibration of the absorption. For the qualitative determination, recording spectrophotometer, Model DK (BECKMAN) was used, and for the quantitative determination, PULFRICH's photometer.

SUMMARY

Separation of the urinary ester-form bilirubin was attempted, and the results obtained may be summarized as follows :

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2. On the basis of the results of paper chromatography and paper electrophoresis, the pigment has been determined to contain no amino acid, steroid, nor reducing substance. Moreover, no glucuronic acid could be detected whether examined *in vitro* or by paper chromatography together with paper electrophoresis, either.

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