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Abstract

Two forms of the direct bilirubin separated from the dried canine cholecystic bile were subjected to paper chromatography and emission or infra-red spectroscopy, and the following results were obtained: 1. The two forms of the direct bilirubin contain plenty of bile acid or its salt, and benzidine- and ninhydrine-positive substances together with various inorganic elements were also detected. 2. The ester-form bilirubin had carboxyl radical by infrared spectroscopy. But it will not be easily concluded that an existence of carboxyl radical will owe to free carboxyl radical of the dibasic acid bilirubin by the reason why an existence of plenty of bile acid in the sample may inhibit the characteristic absorption of ester. 3. It may be suggested that the two forms of the direct bilirubin combine with bile acid or its salt, and that the affinity between them is stronger in the salt-form bilirubin. 4. It seems probable that properties of the salt-form and ester-form bilirubins are not influenced by an existence of bile acid or its salt, and further by acornbination with it.

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STUDIES ON BILE PIGMENTS IV. DIRECT BILIRUBIN IN CANINE BILE

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Since HIJMANS VAN DEN BERGH[1] reported an existence of two kinds of bilirubin, direct and indirect reacting ones, in jaundiced serum towards the EHRLICH's diazo reagent, numerous reports have been made for a contribution to clarify the cause of such a difference, but it seems reasonable to assume that no definite conclusion has been obtained. In view of a series of chemical and clinical experiments in the school of YAMAOKA and his co-workers for years the most reasonable conclusion to be drawn from available data is as follows : The indirect bilirubin which is transferable into chloroform is dibasic acid, while the direct bilirubin which is not transferable into chloroform may be divided, by the attitude towards acid, into two forms, one of which is transferable into chloroform by the addition of acid, but another is not so. The bilirubin transferred into chloroform in the former has all the properties of the indirect bilirubin, and then it is called the salt-form bilirubin. Though the bilirubin in the latter is not transferable into chloroform by acid, it is made so as the dibasic acid bilirubin after saponification with 5% methanolic potash, and it is called the ester-form bilirubin. It is recognizable not only on serum bilirubin, that the direct bilirubin consists of the two forms, but also on urinary and biliary bilirubins[2,3,4]. The above facts will offer a useful aid for clarifying the nature of the different reactivity of serum bilirubin against the EHRLICH's diazo reagent, but a proof of an existence of these two forms entirely depends on a pure isolation of them.

The results of infra-red spectral examination exhibited that the dibasic acid bilirubin treated with NaOH displayed no cha-

racteristic absorption bands of the propionic acid radical, and showed a formation of salt[*1)], which gave the positive diazo reaction in aqueous media. Dimethyl ester of bilirubin prepared by diazomethane as well as dimethyl ester of mesobilirubin prepared by methanol and HCl-gas, on the other hand, also clearly displayed no characteristic absorption bands of the propionic acid radical and abandoned the properties of the dibasic acid bilirubin, and they gave the positive direct diazo reaction in serum after stirring until in solution[*1),*2)]. By comparing the above results with the properties of the two forms of the natural direct bilirubin, the salt-form bilirubin is suggested to agree with bilirubin-salt, especially with sodium salt, and the ester-form one with bilirubin-ester although the nature of these direct bilirubins has not always been cleared up.

SHIMADA[5)] attempted to separate them affording an important base in a separation of the two fractions of the direct bilirubin. The details of the separated two fractions were, however, scarcely mentioned in his report. The author, accordingly, reported a method to separate the indirect bilirubin[6)] and further the two forms of the direct bilirubin[7)] by a modification of SHIMADA's method, and he mentioned the details of the separated bilirubin. It has been shown in his reports that remarkable differences of chemical and spectrochemical properties are generally recognizable among them.

The present investigation was undertaken to clarify whether the two forms of the direct bilirubin may be proved to be in formation of salt or ester in fact or not, and further what causes the essential difference between them.

Experimental

Comparative studies by paper chromatography were undertaken on the crystalline bilirubin, two fractions of the direct bilirubin separated from canine cholecystic bile, their hydrolysates and saponificates being significant for identification of two forms of direct bilirubin, and sodium salts of various bile acids commonly existing in bile. At the same time, detections

*1) SHINDO, M. ; Unpublished data.
*2) HOSOKAWA, M. ; Igaku Kenkyu (in printing).

of inorganic elements in the two fractions of the direct bilirubin as well as surveys on the state of propionic acid radicals at the side chains of bilirubin were undertaken.

1. *Materials.*

1. 1. *Separation of Two Fractions of Direct Bilirubin in Bile.*

The two fractions of the direct bilirubin were separated from chloroform extracts of the dried canine cholecystic bile on silica gel or alumina according to procedures described before[7].

1. 2. *Crystalline Bilirubin.*

The crystalline bilirubin was purified on alumina, and the details were the same as those described in the previous paper in this series[6].

1. 3. *Bile Acid-Salts, Lecithin, and Cholesterol.*

Sodium cholate (TAKEDA), sodium desoxycholate (TAKEDA), sodium glycocholate (MERK), sodium taurocholate (BAYER), lecithin (TAKEDA), and cholesterin (MERK) were availed. These materials were availed for crude samples in methanolic solutions except a chloroform solution of cholesterin.

1. 4. *Hydrolysates of Direct Bilirubins.*

The procedures of HARA[4] were followed: After chloroform was added to a mixture of an equal volume of $N/10$ HCl and an aqueous solution of the direct bilirubin, the mixture was shaken hard ; and then two phases were separated by letting the mixture standing. These two phases were availed for the sample after having concentrated in syrups.

1. 5. *Saponificates of Direct Bilirubins.*

For the procedures followed that of KOSAKA[3] : After the addition of twice volumes of 5% methanolic potash to an aqueous solution of the direct bilirubin, the mixture was boiled for 5 minutes in a boiling water bath ; and then extraction with chloroform was undertaken after the resulting solution had been neutralized with one fifth volume of glacial acetic acid against the sample under cooling in a room. Thus separated chloroform and residual phases were sampled after having concentrated in syrups.

2. *Methods.*

2. 1. *Paper Chromatography.*

2. 1. 1. *Filter Paper.*

TOYO Nos. 2, 3, 7, 50, and 51, supplied from TOYO Filter

Paper Co., AZUMI No. 7, supplied from AZUMI Filter Paper Co., and SCHLEICHER & SCHÜLL (SS) No. 2043 *a*, supplied from SCHLEICHER & SCHÜLL Co., were subjected to selection of the most available paper with reference to the publication of E. LEDERER & M. LEDERER[8], and the last was the most favorable among them in consideration of formation of a brown front due to impurities in the paper, degree and clarity of separation of spots, diffuseness of spots, eveness of the paper, and formation of tailing. But TOYO No. 51 was also partly availed owing to difficulty in acquisition of SS No. 2043 *a*. Filter papers were cut in strips of 40 cm × 5 cm, and starting points were marked in three places at a distance of 1.5 cm from each other on the line of 5 cm from one side of the strip.

2. 1. 2. *Development.*

The paper was availed without preliminary treatment or after treatment with acetic acid or phenol. The treatment means that the paper was immersed in 70 % acetic acid or a phenol, methanol, water mixture (3 : 3 : 2) accompanying drying for 5 minutes at 90°C. Samples were dissolved into methanol or chloroform in 10 mg/dl, and 0.01 ml of them were spotted on the starting point in a spot within 0.5 cm. The paper was hung in a glass cylinder of 10 cm × 45 cm holding a developing solvent, it was let standing for several hours till the cylinder was saturated with the vapor of the solvent, and then the same solvent was further added to immerse about 1 cm of the end of the paper. Development was carried out in a dark room at 12 to 14°C. The paper was dried in a thermostatic incubater at 38°C after development. Though various combinations of several solvents were availed in comparison of chromatograms, the top layer of a *n*-butanol, acetic acid, water mixture (4 : 1 : 5) was often used. This solvent was availed 3 days hence after the preparation.

2. 1. 3. *Identifications of Partitionated Substances.*

Partitionated colour spots were carefully remarked during or immediately after development. Specific colour reactions were undertaken as below :

2. 1. 3. 1. *Detection of Bilirubin.*

2. 1. 3. 1. 1. EHRLICH-VAN DEN BERGH's *Reaction.*

The positive direct reaction was judged from an appearance

of reddish violet spots after spraying the EHRLICH's diazo reagent, and it was identified to the positive indirect diazo reaction what appeared only when a caffeine mixture was further sprayed. This reaction, on the other hand, appeared relatively clearly in comparatively high bilirubin contents, but the colour grew faint or colourless in low concentration.

2. 1. 3. 1. 2. GMELIN *Reaction.*

The GMELIN reaction was availed together with the EHRLICH's diazo reaction to detect the bilirubin on the paper by spraying the reagent, and appearances of violet blue spots were recognized to be positive ones. But no dominant finding could be detected between the two reactions so far as it concerned on the paper.

2. 1. 3. 2. *Detection of Urobilin.*

Appearances of light greenish yellowish fluorescence on the paper under the ultraviolet light means the posibility of an existence of urobilin, and it is further susceptible when the fluorescence is accelerated by spraying the SCHLESINGER's reagent.

2. 1. 3. 3. *Detection of Urobilinogen.*

Urobilinogen was identified by an appearance of a red colour by spraying the EHRLICH's aldehyde reagent.

2. 1. 3. 4. *Detection of Reducing Substances.*

2. 1. 3. 4. 1. PARTRIDGE's *Ammoniacal Silver Nitrate Method.*

After spraying a freshly prepared mixture of a $N/10$ $AgNO_3$ solution and 5 N ammoniac water in equal volume, the paper was heated at 105°C for 5 minutes. The positive reaction was identified by an appearance of light blackish brown spots[9].

2. 1. 3. 4. 2. *Aniline Hydrogen Phthalate Method.*

The positive reaction was identified by an appearance of brown spots by heating the paper at 105°C for 5 minutes after spraying the PARTRIDGE's aniline hydrogen phthalate reagent[10].

2. 1. 3. 4. 3. *Benzidine Reaction.*

The positive reaction was identified by an appearance of brown spots by heating the paper at 105°C for 15 minutes after spraying the HORROCKS' benzidine reagent[11]. Though this reaction is sensitive than the ammoniacal silver nitrate reaction, positive spots may be caused by some amino acids and so on[12].

2. 1. 3. 5. *Detection of Amino Acids.*

2. 1. 3. 5. 1. *Ninhydrine Reaction.*

The reagent was availed as a 0.2 % solution of specially pure ninhydrine in water-saturated *n*-butanol. Colouring was undertaken by heating the paper at 90°C for 10 minutes after spraying the reagent. The reagent is to react upon amino acids, peptides, and proteins which have free amino radical at α position to free carboxyl radical, and it reacts sensitively upon amino acids generally. Identifications of amino acid were further undertaken by following reactions.

2. 1. 3. 5. 2. SAKAGUCHI *Reaction.*

After spraying a 5 % NaOH solution, a 0.1 % alcoholic solution of α-naphthol was sprayed, then, in several minutes, freshly-prepared 5 % sodium hypobromite was further sprayed. The positive reaction was identified by an appearance of red spots.

2. 1. 3. 5. 3. PAULY's *Diazo Reaction.*

A *N* NaOH solution was fully sprayed immediately after spraying a mixture of a saturated solution of sulfanilic acid in 2 % HCl and a 5 % sodium nitrite solution in an equal volume under cooling. The positive reaction was identified by an appearance of orange or reddish orange spots. As similar colouring appears also in cases when ammonia or phenol is availed to developing or immersing solvent, this reaction was omitted then.

2. 1. 3. 5. 4. MILLON's *Reaction.*

A 5% sodium nitrite solution was sprayed 1 hour after spraying a 10% mercuric sulphate solution in 5 *N* H_2SO_4. The positive reaction was identified by an appearance of red spots.

2. 1. 3. 5. 5. *Iodine-Azide Reaction.*

When a mixture of a *N*/20 I-KI solution and a 5% sodium azide solution in an equal volume was sprayed, an appearance of white spots against brown ground was recognized as specific ones.

2. 1. 3. 6. *Detection of Bile Acids.*

2. 1. 3. 6. 1. *Antimony Trichloride Reaction.*

After immersing the paper in a 20 % $SbCl_3$ solution in chloroform, the paper was heated at 80°C for 3 to 5 minutes. An appearance of violet red fluorescent spots under the ultraviolet light and violet red colours at the same spots were recognized to be specific. This reaction is well used for detection of carotinoids and steroids.

2. 1. 3. 6. 2. *Phosphomolybdic Acid Reaction.*

After spraying a 10% alcoholic phosphomolybdic acid solution, the paper was heated at 80° to 100°C for 5 minutes. An appearance of greenish blue spots was recognized to be specific.

2. 1. 3. 6. 3. *Phosphoric Acid Reaction.*

After spraying a 15% aqueous phosphoric acid solution, the paper was heated at 80°C for 5 minutes. An appearance of light violet red spots and yellowish violet fluorescence at the same place were recognized to be specific.

2. 2. *Emission Spectroscopy.*

Qualitative analyses to detect a minute amount of inorganic elements in the sample by calibrating spectral lines were carried out with Quartz Spectrophotometer Model QF 60 (SHIMADZU). A collimating lens was arranged following that of the intermediate image method, and spark was discharged by Feussner-type equipment under conditions of A. C. 90 V of the primary voltage, 15, 000 V of the secondary voltage, 0.8 millihenry of self inductance, and 0.005 mm of slit. Photography was undertaken after 30 minutes' prespark, and spectrum was photographed on process hard plate (4' × 10') prepared for spectroanalytical use (SHIMADZU) at 2 minutes' exposure. Samples were set in a form of powders or solutions into a small hole of 2.5 mm × 2.5 mm (about 0.01 ml) of pure carbon electrode which was supplied for spectroanalytical use from SHIMADZU Co. The carbon electrode was dried in a thermostatic incubator at 75°C for 3 hours after offering the sample, and then it was availed as the lower electrode.

2. 3. *Estimation of Infra-Red Absorption Spectra.*

Infra-red absorption was estimated on powders of the two fractions of the direct bilirubin to study whether carboxyl radicals were present or not, and further whether formation of ester was recognized or not. Calibration was carried out with PERKIN ELMER 12 C, and samples were offered by the nujol-paste method.

Results

As PARTRIDGE's ammoniacal silver nitrate and aniline hydrogen phthalate reactions for reducing substances, and SAKAGUCHI,

PAULY's diazo, iodine-azide, and MILLON's reactions for amino acids were negative so far as examined, the details of the results by these reactions are omitted hereafter.

1. *Paper Chromatoyraphy.*

1. 1. *Paper Chromatograms of Crystalline Bilirubin.*

When development was carried out with solvents containing ammonia, phenol, lutidine, or collidine under the stationary phase of water, tailing of bilirubin was remarkable. In such cases, light greenish yellow spots were partitionated diffusely somewhere. When stationary phase was phenol, bilirubin was partly partitionated, and the colour was light greenish yellow, but a greater part was left on the starting point showing an orange yellow colour. Thus, bilirubin was apt to remain on the starting point in spite of using various developing solvents and different stationary phases, and no other specific spot could be detected on the paper except the GMELIN and indirect diazo reactions (Fig. 1).

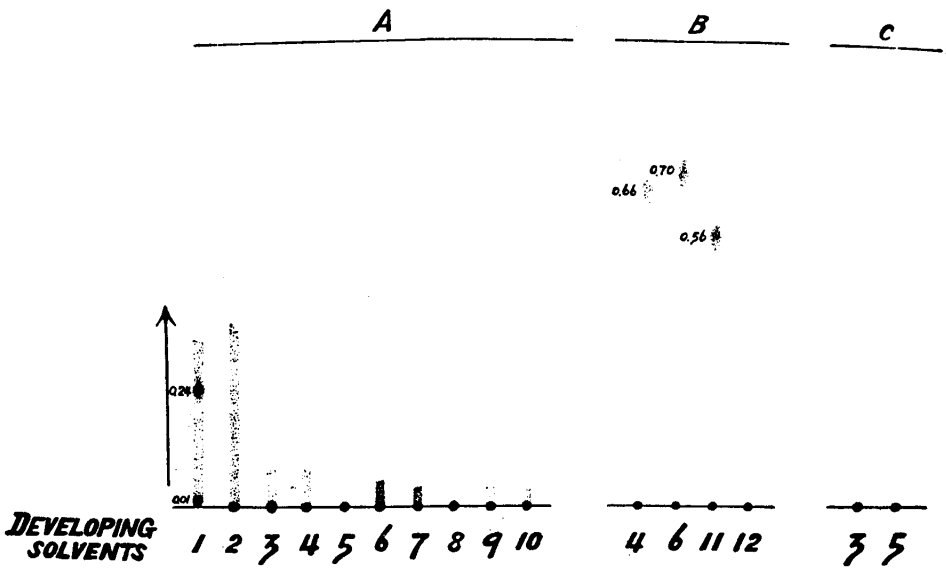

Fig. 1. Paper chromatograms of indirect bilirubin

Sample :	Crystalline bilirubin, ca. 0.1 mg.
Paper :	TOYO No. 51.
Method :	One-dimensional ascending method.
Temperature :	12—14°C.
Run :	30—35 cm.
Colouring :	None.

Abbr. Treatment of papers :

A·····No treatment.
B·····Phenol-treated papers.
C·····Acetic acid-treated papers.

Developing solvents :

1·····*n*-Butanol, ethanol, ammonia, water mixture (40 : 10 : 1 : 49)
2·····Methanol, ammonia, water mixture (15 : 1 : 4)
3·····*n*-Butanol, acetic acid, water mixture (4 : 1 : 5)
4·····*n*-Butanol, phenol, water mixture (4 : 1 : 5)
5·····*n*-Butanol, ethanol, water mixture (4 : 1 : 5)
6·····*n*-Butanol, lutidine, water mixture (4 : 1 : 5)
7·····*n*-Butanol, collidine, water mixture (4 : 1 : 5)
8·····Butyl acetate, acetic acid, water mixture (10 : 1 : 5)
9·····Butyl acetate, ethanol, water mixture (10 : 1 : 5)
10·····Water-saturated butyl acetate
11·····*n*-Propanol, phenol, water (10 : 1 : 9)
12·····*n*-Propanol, ethanol, Water (5 : 2 : 5)

1. 2. *Paper Chromatograms of Salt-Form Bilirubin.*

A diffuse yellow spot tailing towards the front was found in the salt-form bilirubin when the main constituent of developing solvents was alcohol. The spot corresponded to bilirubin, bile acids, reducing substances, and amino acids ; and one or two spots corresponding to bile acids were found on the tail by the phosphomolybdic acid reaction together with bilirubin, but there happened to occur an atypical brownish violet spot on the tail by the $SbCl_3$ reaction in case of prolongation of heating. Benzidine positive spots were found not only always at the place described above, but also often at lower Rf values showing a light brown spot on the brown paper. Ninhydrine-positive spots were mostly agreed with the benzidine-positive ones (Figs. 2, 3).

For example : A yellow tone was most intense at Rf 0.35 to 0.4 accompanying a yellow tail till the front when the top layer of a *n*-butanol, acetic acid, water mixture (4 : 1 : 5) was availed to developing solvent as well as availing water to the stationary phase. A spot at Rf 0.35 to 0.4 gave the positive GMELIN reaction, but neither the EHRLICH's diazo reaction, the EHRLICH's aldehyde reaction, nor the SCHLESINGER's reaction was positive there. Phosphomolybdic acid-positive spots were found at Rf 0.39 to 0.41, 0.46 to 0.50, and 0.78, $SbCl_3$-positive ones at Rf 0.38 to 0.41 and 0.46 to 0.50, and phosphoric acid-positive

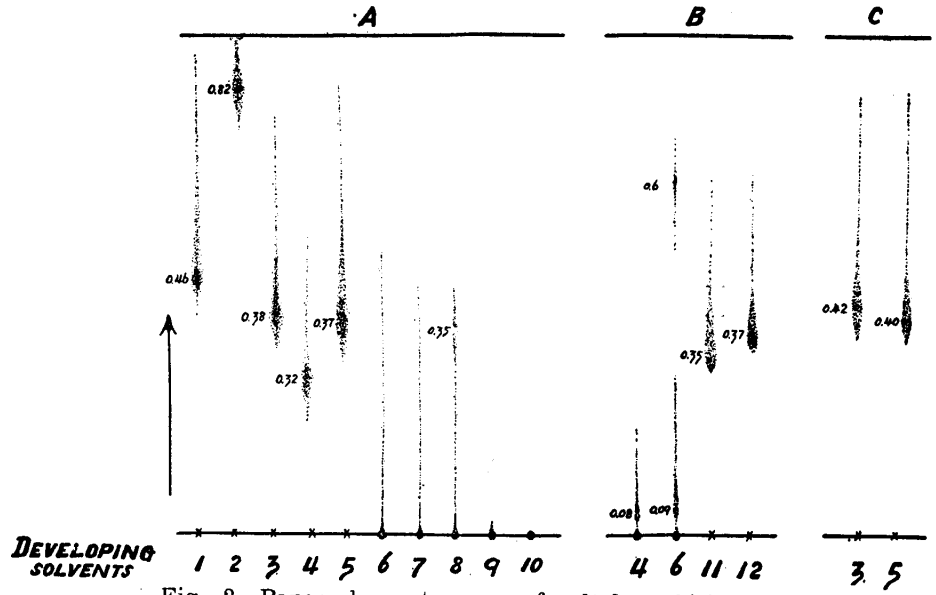

Fig. 2. Paper chromatograms of salt-form bilirubin
Sample : Salt-form bilirubin, ca. 0.1 mg.
Paper : TOYO No. 51.
Method : One-dimensional ascending method.
Temperature : 12—14°C.
Run : 30—35 cm.
Colouring : None.
Numbers in chromatograms are mean values of Rf values of each spot.

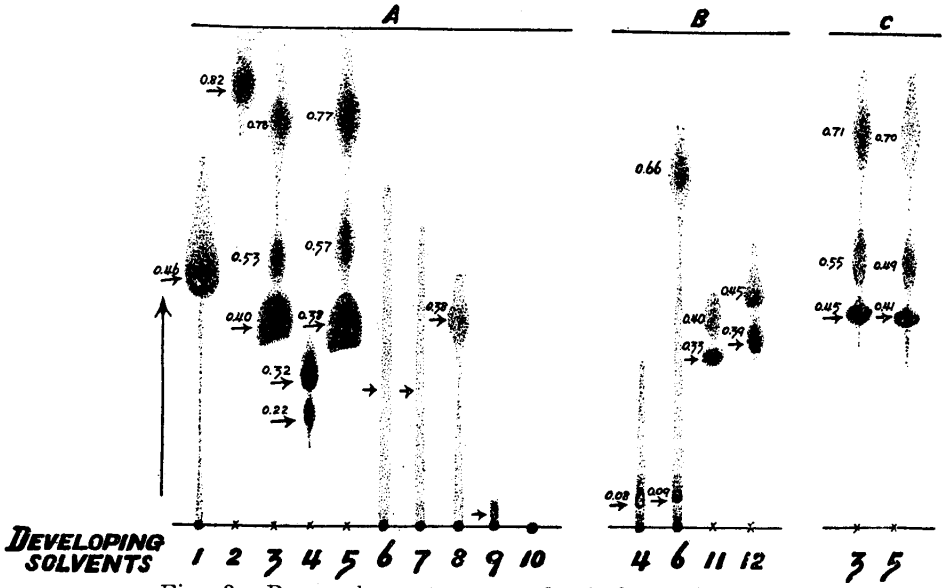

Fig. 3. Paper chormatograms of salt-form bilirubin
Chromatograms were developed under the same condition as that of Fig. 2 except that colouring was carried on with the phosphomolybdic acid method.
Abbr. →..... Phosphoric acid-positive spots.

ones at Rf 0.38 to 0.40 respectively. Benzidine-positive spots were found at Rf 0.36 to 0.41 and 0.46 to 0.51, and ninhydrine-positive ones at Rf 0.39 to 0.40, 0.27 to 0.28, and 0.20 to 0.22 (Tables 1—5).

1. 3. *Paper Chromatograms of Ester-Form Bilirubin.*

Similar chromatograms were developed with the same solvents as in the salt-form bilirubin, but a diffuse yellow spot was partitionated at a lower Rf value in the ester-form bilirubin showing scarce tailing towards the front contrary to a clear tailing till the starting point. The spot gave an undistinguished GMELIN reaction together with neither the EHRLICH's diazo reaction, the EHRLICH's aldehyde reaction, nor the SCHLESINGER's reaction. GMELIN-positive spots could be found at neither the tail nor the start. Bile acid was partitionated at two or three places, one of which was found at a slightly distant place from the yellow spot towards the front showing a far faint colour than that of the salt-form bilirubin, and the other bile acid spots were found between the former and the starting point although these spots gave far weaker colours than the former. Benzidine-

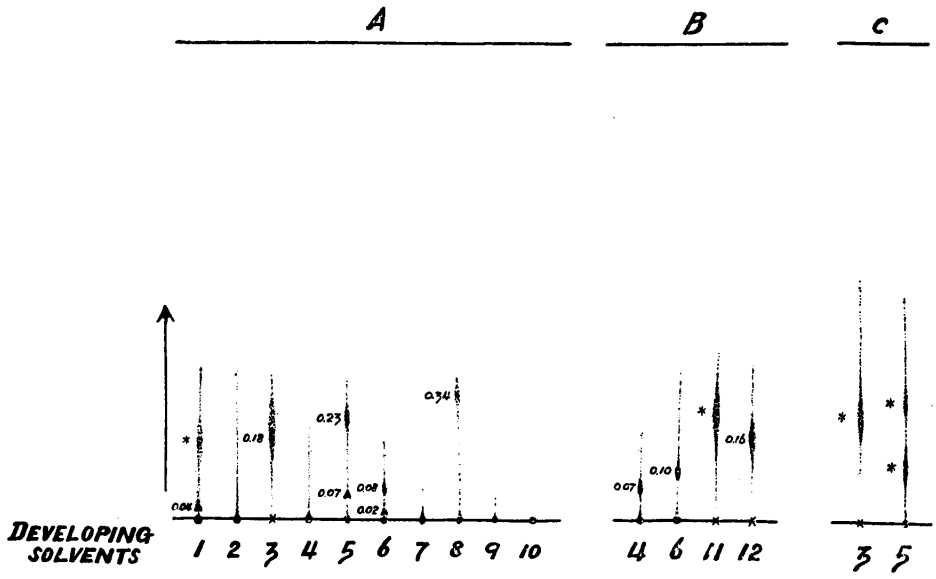

Fig. 4. Paper chromatograms of ester-form bilirubin

Chromatograms were developed under the same condition as that of Fig. 2 except that about 0.1 mg of the ester-form bilirubin was sampled.
Abbr. *.....Tailing was too remarkable to calibrate the Rf value.

positive spots were found to correspond to bile acid spots, especially the upper one, and the yellow spot was also found to correspond to bilirubin, bile acid, reducing substances, and further amino acids although it was found sometimes as a white spot against a light brown ground (Figs. 4, 5).

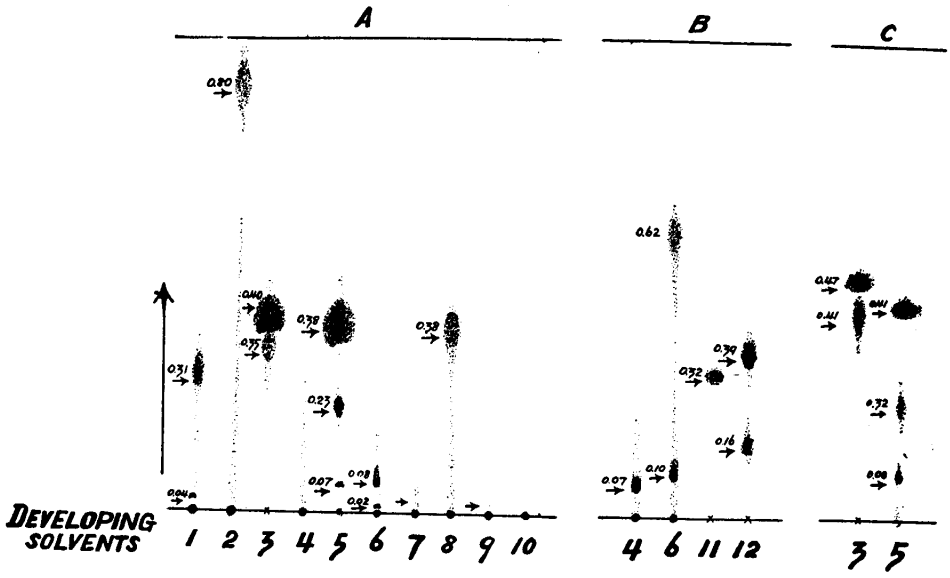

Fig. 5. Paper chromatograms of ester-form bilirubin

Chromatograms were developed under the same condition as that of Fig. 4 except that colouring was carried on with the phosphomolybdic acid method.

For example : A paper chromatogram of the ester-form bilirubin, under development with the top layer of a *n*-butanol, acetic acid, water mixture (4 : 1 : 5) availing water as the stationary phase, had a comparatively intense yellow spot at Rf 0.18 to 0.21, and tailing from there was recognized upwards and downwards till Rf 0.35 to 0.40 and the starting point. The yellow spot of Rf 0.18 to 0.21 gave the fairly positive GMELIN reaction, but not the remarkable diazo reaction, and neither the EHRLICH's aldehyde reaction nor the SCHLESINGER's reaction. Bile acid was detected at Rf 0.36 to 0.41 and borderingly at Rf 0.34 to 0.37, and the spot was more intense in the former than in the latter, and the spot of the former was far weaker than that of

Rf 0.39 to 0.41 in the salt-form bilirnbin. Benzidine-positive spots were found at Rf 0.18 to 0.20 clearly and sometimes in a light brownish spot against a brown ground together with the one at Rf 0.35 to 0.40. A ninhydrine-positive spot was found at Rf 0.21 to 0.22 agreeing with the yellow spot of Rf 0.18 to 0.21 (Tables 1—5).

Table 1

Rf values of benzidine-positive spots of direct bilirubin and bile acid-salts

Chromatograms were developed on SS No. 2043 *a* filter papers with the top layer of a *n*-butanol, acetic acid, water mixture (4 : 1 : 5) by a one-dimensional ascending method. About 0.1 mg of the sample was availed respectively. The spraying reagent was sprayed on the paper after an about 30—35 cm run.

Samples	Rf values		
Salt-form bilirubin	0.48*1) (0.46-0.51)	0.38 (0.36-0.41)	
Ester-form bilirubin		0.36 (0.35-0.40)	0.19*2) (0·18-0.20)
Sodium cholate		0.30 (0.29-0.38)	0.16*2)*3) (0.15-0.18)
Sodium desoxycholate			0.18*2) (0.18-0.21)
Sodium taurocholate	0.47 (0.44-0.50)	0.35 (0.34-0.37)	0.17*2)*3) (0.15-0.18)
Sodium glycocholate		0.39 (0.36-1.41)	0.19*2)*3) (0.15-0.21)
Lecithin			
Cholesterol	0.86 (0.77-0.92)		

*1) Values are mean values, and those in parentheses mean fluctuated ones.
*2) Light brown spots against brown grounds.
*3) Sometimes unrecognizable.

Table 2.
Rf values of phosphomolybdic acid-positive spots of direct bilirubin and bile acid-salts

Samples	Rf values				
Salt-form bilirubin	0.78*1) (0.78)	0.48 (0.46-0.50)	0.39 (0.39-0.41)		
Ester-form bilirubin			0.40*2) (0.36-0.41)	0.35*3) (0.34-0.37)	0.19*4) (0.18-0.21)
Sodium cholate	0.79*1) (0.78-0.80)	0.41 (0.40-0.42)	0.30 (0.30-0.32)		
Sodium desoxycholate	0.80*1) (0.79-0.82)				0.19*4) (0.18-0.21)
Sodium taurocholate		0.46 (0.45-0.47)	0.34 (0.33-0.35)		
Sodium glycocholate		0.46 (0.44-0.47)	0.35 (0.33-0.35)		
Lecithin	0.86*1) (0.84-0.88)				
Cholesterol					

*1) Remarkably tailed.
*2) Very weakly coloured spots to compare with those of the salt-form bilirubin.
*3) Far weaker spots to compare with Rf 0.40 spots.
*4) White fleck.

Table 3.
Rf values of antimony trichloride-positive spots of direct bilirubin and bile acid-salts

Samples	Rf values			
Salt-form bilirubin		0.48 (0.46-0.50)	0.39 (0.38-0.41)	
Ester-form bilirubin			0.37 (0.36-0.41)	0.35 (0.34-0.37)
Sodium cholate			0.4	0.3
Sodium desoxycholate	0.8*1)			
Sodium taurocholate		0.45	0.3-0.4	
Sodium glycocholate		0.45	0.3-0.4	
Lecithin	0.8-0.9*1)			
Cholesterol	0.9*2)			

*1) Sometimes recognizable as atypical brown spots.
*2) Atypical brown spots at the frontal region.

Table 4.

Rf values of phosphoric acid-positive spots of direct bilirubin and bile acid-salts

Samples	Rf values	
Salt-form bilirubin	0.39 (0.38-0.40)	
Ester-form bilirubin	0.38 (0.36-0.41)	0.36 (0.34-0.37)
Sodium cholate	0.3	
Sodium desoxycholate		
Sodium taurocholate	0.3-0.4	
Sodium glycocholate	0.3-0.4	
Lecithin		
Cholesterol		

Table 5.

Rf values of ninhydrine-positive spots of direct bilirubin and bile acid-salts

Samples	Rf values			
Salt-form bilirubin	0.39 (0.39-0.40)	0.27 (0.27-0.28)	0.22 (0.20-0.22)	
Ester-form bilirubin			0.21 (0.21-0.22)	0.16* (0.15-0.17)
Sodium cholate	0.41* (0.40-0.41)	0.31* (0.28-0.32)	0.17 (0.15-0.19)	
Sodium desoxycholate			0.18 (0.17-0.21)	
Sodium taurocholate	0.36* (0.33-0.38)		0.19 (0.17-0.20)	0.12 (0.11-0.13)
Sodium glycocholate			0.21 (0.19-0.22)	0.14 (0.13-0.16)
Lecithin				
Cholesterol				

* Weakly positive spots.

Each spot had a violet colour, and no spot gave the positive SAKAGUCHI, PAULY's diazo, MILLON, or iodine-azide reaction.

1. 4. *Paper Chromatomatograms of Hydrolysates of Direct Bilirubins.*

By hydrolysis with acid, according to the procedure followed that of HARA, all the yellow pigment in the salt-form bilirubin was seen to be transferred into the chloroform phase leaving no colour behind in the residual phase. To compare the substances in each phase on the paper under development with the top layer of a *n*-butanol, acetic acid, water mixture (4 : 1 : 5) utilizing water as the stationary phase, the yellow pigment of the chloroform phase was most intensely partitionated at Rf 0.30 to 0.36 accompanying a clear tail upwards to the front and slightly downwards to the starting point, while a faint yellow or light brownish spot was found at Rf 0.32 to 0.34 in the residual phase. The GMELIN and indirect diazo reactions were given at Rf 0.30 to 0.36 in the case of chloroform phase, but both the EHRLICH's aldehyde and SCHLESINGER's reactions were not given anywhere. None of these reactions was positive at Rf 0.32 to 0.40 in the case of the residual phase. In the case of chloroform phase, bile acid was weakly detected at Rf 0.32 by three bile acid-detecting reactions and at Rf 0.42 by two reactions except the phosphoric acid reaction very faintly, and benzidine-positive spots were found indistinctly at Rf 0.19 and 0.03, but no ninhydrine-positive spot was found. And in the case of the residual phase, bile acid was detected at Rf 0.39 by the aforementioned three reactions and at Rf 0.48 by the two reactions except the phosphoric acid one, but an atypical phosphomolybdic acid-positive spot could be found from Rf 0.8 to the front. Ninhydrine-positive spots were seen at Rf 0.44, 0.38, 0.21, and 0.15, where no colour appeared other than violet.

Almost no yellow pigment was seen to be transferred into chloroform by hydrolysis of the ester-form bilirubin. By developing the two phases with the same developing solvent as in the salt-form bilirubin, a yellow tone was recognized at the front faintly in the chloroform phase and remarkably in the residual phase. No specific spot corresponding to bilirubinoid, bile acid, reducing substance, or amino acid was found in the chloroform phase. The residual phase, on the other hand, gave no bilirubinoid at any place, bile acid at Rf 0.33, benzidine-positive spots unclearly at Rf 0.16 and indistinctly at Rf 0.38, and

ninhydrine-positive spots clearly at Rf 0.16 and slightly at Rf 0.21 (Table 6).

Table 6.

Rf values of benzidine-, antimony trichloride-, and ninhydrine-positive spots of hydrolysates of direct bilirubin

Samples		Benzidine	$SbCl_3$	Ninhydrine
Salt-form bilirubin	$CHCl_3$ ext.	— — (0.19) (0.03)	— (0.42) (0.32)	— — — —
	Residual	(0.50) 0.41 (0.19) —	0.48 0.39 —	0.44 0.38 0.21 0.15
Ester-form bilirubin	$CHCl_3$ ext.	— — — —	— — —	— — — —
	Residual	— (0.38) 0.16 —	— — 0.33	— —(0.21) 0.16

Spots of parenthesized values were dim.
Chromatograms were developed on SS No 2043 *a* filter papers with the top layer of a *n*-butanol, acetic acid, water mixture (4 : 1 : 5).

1. 5. *Paper Chromatograms of Saponificates of Direct Bilirubins.*

When the salt-form bilirubin was extracted into chloroform after saponification of it, the yellow pigment was partly transferred into chloroform of which a greater part remaining into the residual phase, and these two phases were chromatographed under development with the top layer of a *n*-butanol, acetic acid, water mixture (4 : 1 : 5) utilizing water as the stationary phase. In the chloroform phase, a yellow tone moved to the front showing no good partition, and the yellow pigment was not identified to bilirubinoid by the aforementioned reactions. Bile acid was detected at the same place by the above three reactions, but there appeared no spot corresponding to bile acid, reducing substance, and amino acid except the above place. In the residual phase, a yellow tone was most apparent at Rf 0.3 to 0.4 although it tailed from there upwards and downwards. At least, the yellow pigment could not be identified to bilirubinoid on the paper. Bile acid spots were detected at Rf 0.36 by the aforementioned three reactions, and at Rf 0.48 by two reactions except the phosphoric acid one. Benzidine-positive spots were detected at Rf 0.45 indistinctly, 0.36, and 0.18 indistinctly, but ninhyd-

rine-positive ones were found at Rf 0.33, 0.16, and 0.10 dimly.

The yellow pigment of the ester-form bilirubin was almost transferred into chloroform leaving a little colour behind the residual phase by saponification, and these two phases were similarly chromatographed to the above. A yellow tone moved to the front, where no bilirubinoid could be detected so far. Two benzidine-positive spots were detected at Rf 0.98 and 0.94 in the case of the chloroform phase, but there was found no spot corresponding to bile acid or amino acid (Table 7).

Table 7.

Rf values of benzidine-, antimony trichloride-, and ninhydrine-positive spots of saponificates of direct bilirubin

	Samples	Benzidine				$SbCl_3$			Ninhydrine		
Salt-form bilirubin	$CHCl_3$ ext.	0.98	—	—	—	0.96 (0.90)	—	—	—	—	—
	Residual	—	(0.45)	0.36	(0.18)	—	0.48	0.36	(0.33)	(0.16)	(0.10)
Ester-form bilirubin	$CHCl_3$ ext.	0.98 (0.94)	—	—	—	—	—	—	—	—	—
	Residual	—	—	—	—	—	—	—	—	—	—

Spots of parenthesized values were dim.
Development was the same as in Table 6.

1. 6. *Paper Chromatograms of Bile Acids, Lecithin, and Cholesterol.*

Similar chromatograms were developed with the top layer of a *n*-butanol, acetic acid, water mixture (4 : 1 : 5) utilizing water as the stationary phase when sodium salts of cholic, taurocholic, and glycocholic acids were sampled. Sodium cholate was recognized at about Rf 0.3 showing the above three bile acid reactions and a faint ninhydrine reaction, at about Rf 0.4 showing the two bile acid reactions except the phosphoric acid one, a faint ninhydrine reaction at about Rf 0.8 showing the phosphomolybdic acid reaction, and at about Rf 0. 15 showing benzidine and ninhydrine reactions. Sodium desoxycholate was partitionated tailing at Rf 0.8 by the phosphomolybdic acid reaction slightly, and by the benzidine reaction undistinguishedly. Sodium taurocholate or glycocholate gave spots correspon-

ding to bile acid between Rf 0.3 and 0.4, and a faint ninhydrine-positive spot was also detected in the former. Further, phosphomolybdic acid- and $SbCl_3$-positive spots were found at about Rf 0.45, and sodium taurocholate gave a benzidine-positive spot here. There appeared faint benzidine- and ninhydrine-positive spots at about Rf 0.2, and further a ninhydrine-positive spot was also found at Rf 0.13. Lecithin gave a phosphomolyb-

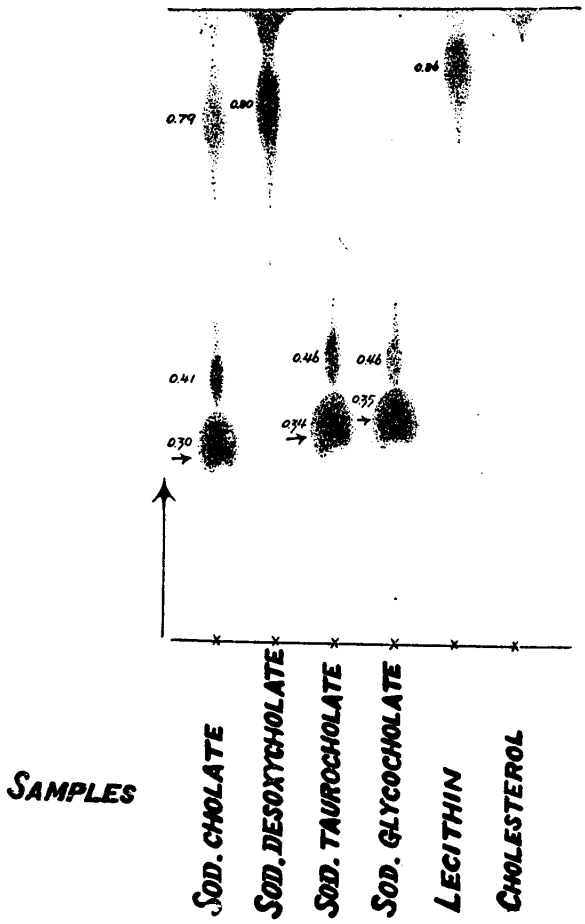

Fig. 6. Paper chromatograms of bile acid-salts
Sample : About 0.1 mg respectively.
Paper : TOYO No. 51.
Method : One-dimensional ascending method.
Temperature : 12—14°C.
Run : 30—35 cm.
Colouring : Phosphomolybdic acid.
Developing solvent : *n*-Butanol, acetic acid, water mixture (4 : 1 : 5)

dic acid positive spot at Rf 0.85 alone, and cholesterol a benzidine-positive one at Rf 0.9 (Tables 1—5, Fig. 6).

2. *Emission Spectroscopy.*

Detection of Na was judged from the spectral line at 3302.2 Å, K at 3440.4 Å, Ca at 3158.9 Å, Mg at 2936.5 Å, Cu at 3247.5 Å, and P at 2535.6 Å. No Na, K, or Ca was always detected from the sample of 10 mg or less of the salt-form bilirubin within the detectable limits, but Mg and P were detected from the sample of 1 mg or more of it. No Na, K, P, Mg, or Ca was detected from the sample of 1 mg of the ester-form bilirubin within the detectable limits, but Cu was always detected (Table 8).

Table 8.

Inorganic elements detected from direct bilirubin by emission spectroscopy

Samples	Na	K	Mg	Ca	P	Cu
Electrode test	—	—	—	—	—	—
Salt-form bilirubin (10mg)	—	—	+	—	++	—
Salt-form bilirubin* (10% aqueous solution)	—	—	±	—	+	—
Salt-form bilirubin* (1% aqueous solution)	—	—	—	—	—	—
Ester-form bilirubin* (10% aqueous solution)	—	—	—	—	—	+
Mixture of 0.1 *M* NaCl and 1 *M* P_2O_5*	+++	—	—	—	+++	—

* 0.01 ml was sampled.

3. *Infra-Red Absorption Spectrum.*

The ester-form bilirubin had free carboxyl radicals and infra-red absorption of the ester could not be detected.

Discussion

The natural direct bilirubin has a characteristic property of bilirubin in giving the GMELIN and EHRLICH's diazo reactions forming the typical azobilirubin, which is undoubtedly trans-

formable into HCl-azobilirubin, whether it may exist in serum, bile, or urine. But the two forms of the direct bilirubin differ physically and spectrochemically from the indirect dibasic acid bilirubin from the points of transferability into definite solvents, difference of absorption maxima, and different attitude towards the EHRLICH's diazo reagent[2,6,7]. The direct bilirubin thus proving characteristic may be further divided into two forms, one is easily transferable into chloroform as the dibasic acid bilirubin by the addition of acid, and the other is so only after saponification with methanolic potash[3,4]. This phenomenon has been hitherto understood to owe to hydrolysis of alkali salts of bilirubin into the didasic acid bilirubin in the salt-form bilirubin, and to saponification of bilirubin-ester into the dibasic acid bilirubin in the ester-form bilirubin[2,3,4]. This explanation may be concordant, as described in the introduction, to experiments that synthesized alkali salts or dimethyl esters of bilirubin react upon the EHRLICH's diazo reagent directly in aqueous or serum solutions[*1,*2].

Though the salt-form or ester-form bilirubin availed here was extraordinarily hygroscopic amorphous substance to compare with the similarly-prepared dibasic acid bilirubin, none of them was dissolved into chloroform contrary to being easily soluble into water and alcohol, and the salt-form bilirubin had an absorption maximum at 420—430 mμ in an alcoholic solution and the ester-form one at 415 mμ in an aqueous or alcoholic solution comparing with the dibasic acid bilirubin having the maximum at 450 mμ in a chloroform solution. The EHRLICH's diazo reagent reacted upon these two forms of the direct bilirubin directly. The salt-form bilirubin was easily transferable into chloroform by adding acid, but it was not influenced by a saponification procedure. The ester-form bilirubin was quite contrary to the salt-form in the above phenomenon[7]. These properties were well agreed with those of synthesized salts or esters of bilirubin in having the properties of the direct bilirubin.

Paper chromatograms of the two forms of the direct bilirubin differed remarkably from those of the crystalline bilirubin, and the views of partition differed also between the two forms of the direct bilirubin. Though bilirubin could not be detected in many cases by the GMELIN and the direct or indirect diazo

reactions so far as on the paper, this may owe to less bilirubin content of the sample. But no other bilirubinoid could be also detected so far as concerned. From the view point of phosphomolybdic acid-positive spots, bilirubin spots almost always agreed with phosphomolybdic acid-positive spots judging from partition of bilirubin, but the latter spots were not always concurred to the former ones judging from partition of phosphomolybdic acid-positive ones, and further bile acid was, whether both spots agreed well or not, far largely contained in the spot than bilirubin was. Then, bilirubin content of the sample was calibrated by the procedure of HARA[4], and it was found that bilirubin content was merely 1/500 of the sample. Special regard must be paid to this fact on judgement of the results of both emission spectroscopy and infra-red absorption spectrum.

By clarifying these details, detections of bile acid, reducing substances, and amino acid on paper chromatograms of both salt-form and ester-form bilirubin were compared with those of bile acid-salts, lecithin and cholesterol under development with the top layer of a *n*-butanol, acetic acid, water mixture (4 : 1 : 5) utilizing water as the stationary phase. Though bile acid-detecting reactions applied here are generally used for detections of steroid, detectability of bile acid from the samples is quite within the bounds of possibility than that of the other sterod is. This may permit the reactions to be concluded to be specific to bile acids together with the reason why all the positive spots agreed well with those for control. Thus, the availing samples contained plenty of bile acid-salts against bilirubin, but it is a quite important matter to conclude whether these bile acid-salts combine with bilirubin or not. Spots of the salt-form bilirubin always and those of the ester-form one almost always agreed with those of phosphomolybdic acid under various stationary phases and developing solvents as described before, but some questions occur after surveying the chromatogram under development with the top layer of a *n*-butanol, acetic acid, water mixture (4 : 1 : 5) utilizing water as the stationary phase. Though the salt-form bilirubin was partitionated at Rf 0.35 to 0.4 and there appeared benzidine-positive and ninhydrine-positive spots as well as bile acid-positive one there, the ester-form bilirubin was partitionated at Rf 0.18 to

0.21 where a ninhydrine-positive spot was detected, but typical bile acid was recognized at Rf 0.36 to 0.4 accompanying a benzidine-positive spot there, and this chromatogram agreed with that of the salt-form bilirubin and further with that of cholate, taurocholate, or glycocholate in view of bile acid. But sodium desoxycholate had a phosphomolybdic acid-positive spot at Rf 0.8 and ninhydrine-, traced white phosphomolybdic acid-, and benzidine-positive spots at about Rf 0.2, and this may well be judged to have an intimate relation between the ester-form bilirubin and sodium desoxycholate. But this possibility will be doubted by the reason why the main spot of sodium desoxycholate is at Rf 0.8 and the ester-form bilirubin is not partitionated there, but the main spot of the latter lies at Rf 0.18 to 0.21 where a certain substance, that gives a particular attitude towards the benzidine or phosphomolybdic acid reaction, might be hidden relating to the ester-form bilirubin. And further, it is not well explained merely from the above results whether the salt-form bilirubin combines with bile acid of cholate group or they had similar Rf values by chance, but it may be more reasonable to conclude that the salt-form bilirubin is in combination with a certain bile acid of cholate group from paper chromatograms under development with various solvents, when it becomes an important matter whether the properties of the direct bilirubin will be decided by the combined bile acid or not. According to YAMAMOTO[*3)], it necessitates 100 times or more in mole numbers of bile acid when the dibasic acid bilirubin is dissolved into an aqueous solution of bile acid, and then the EHRLICH's diazo reagent reacts upon the bilirubin atypically, and its absorption maximum shifts to 455 mμ contrary to 450 mμ of the dibasic acid bilirubin in a chloroform solution. These results are quite different from the samples in proving the maxima at 420—430 mμ or 415 mμ. In view of the data in our department it is suggested that an influence of bile acid on the absorption maxima of the dibasic acid bilirubin appears on a slight shift towards the longer wavelength.

It was also surveyed what the appearance of benzidine- and ninhydrine-positive spots means. The benzidine reaction is reported to be the most sensitive one among the reducing sub-

*3) YAMAMOTO, S. ; Unpublished data.

stance-detecting reactions, and further that it may react upon a sort of amino acids. But in the present results, it appeared alone accompanying no other reducing substance-detecting reaction, and it appeared at the same place where bile acid could be recognized. Amino acid was detected only by the ninhydrine reaction accompanying no other spot except faint benzidine-positive spots. And then, it is also uncertain whether these spots mean an existence of reducing substances and breakdown products of protein alone or they owe to taurine or glycine as a component of bile acid. But it may be significant that these spots are similarly partitionated among the samples and the controls.

For the purpose of clarifying the relationship between the salt-form or esterform bilirubin and bile acid or other detected substances, it was surveyed whether these substances will be influenced by the aforementioned procedures classifying the two forms of the direct bilirubin, and examinations were carried out under development with the top layer of a *n*-butanol, acetic acid, water mixture (4 : 1 : 5) utilizing water as the stationary phase. Acidification of the salt-form bilirubin seemed to have made the bilirubin almost transferred into chloroform leaving almost no colour behind in the residual phase, but the bilirubin transferred into chloroform was partitionated at Rf 0.3 to 0.36 where bile acid was also coordinatively recognized, though faint, contrary to the chromatograms of the indirect dibasic acid bilirubin. Chromatograms of the residual phase had a yellow tone at Rf 0.32 to 0.4, and bile acid-, benzidine-, and ninhydrine-positive spots were also similarly detected to those before the procedure. And then, the difference of partition, between the dibasic acid bilirubin and the salt-form bilirubin in view of bilirubin, and between the chloroform phase and the residual phase after hydrolysis of the salt-form bilirubin in view of bile acid, will prove an existence of combination between the bilirubin and bile acid in the salt-form bilirubin. The ester-form bilirubin was found to receive no change by acidification contrary to the salt-form one showing no transferability of yellow pigment into chloroform, and no substance in the chloroform phase was partitionated except the frontal traced yellow pigment. Chromatograms of the residual phase quite resembled

to those of before the procedure except the frontal yellow colour. Therefore, the ester-form bilirubin differed from the salt-form bilirubin and further from the crystalline bilirubin in relation to bile acid and bilirubin itself.

The salt-form bilirubin was transferred partly into chloroform by saponification leaving the greater part of the bilirubin behind in the residual phase, and the chloroform phase was partitionated at the front where bile acid spots and benzidine-positive ones were recognized, but chromatograms of the residual phase agreed with those of before the procedure except a yellow tailing till the startinrg point. Therefore, bilirubin transferred into chloroform from the salt-form bilirubin differs from each other by whether the transferation owes to acidification or saponification. Almost all the bilirubin in the ester-form bilirubin was seen to be transferred into chloroform by saponification, and chromatograms of the chloroform phase had yellow spots at the frontal region where benzidine-positive spots were only recognized. No favorable result could be obtained for an explanation of the reason why no significant spot was partitionated from both phases after saponification of the ester-form bilirubin, and it is not concluded whether this may owe to less bilirubin content of the sample or an influence of saponification.

Above mentioned results show what the salt-form or the ester-form bilirubin has a respective property even if bile acid be concerned.

Summary

Two forms of the direct bilirubin separated from the dried canine cholecystic bile were subjected to paper chromatography and emission or infra-red spectroscopy, and the following results were obtained :

1. The two forms of the direct bilirubin contain plenty of bile acid or its salt, and benzidine- and ninhydrine-positive substances together with various inorganic elements were also detected.

2. The ester-form bilirubin had carboxyl radical by infra-red spectroscopy. But it will not be easily concluded that an existence of carboxyl radical will owe to free carboxyl radical

of the dibasic acid bilirubin by the reason why an existence of plenty of bile acid in the sample may inhibit the characteristic absorption of ester.

3. It may be suggested that the two forms of the direct bilirubin combine with bile acid or its salt, and that the affinity between them is stronger in the salt-form bilirubin.

4. It seems probable that properties of the salt-form and ester-form bilirubins are not influenced by an existence of bile acid or its salt, and further by a combination with it.

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