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The Excretion of Bilirubin as a Diglucuronide Giving the Direct van den Bergh Reaction

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van den Bergh & Müller (1916) reported that there was a difference between bilirubin and the bilirubin-like pigment which is excreted in bile. They observed that bilirubin, and the serum pigment of patients with haemolytic jaundice, required ethanol for coupling with diazotized sulphanilic acid in acid solution. These materials were said to give an 'indirect' reaction. In contrast, the pigment of bile, and of sera from patients with obstructive jaundice and hepatitis, reacted 'directly', without the addition of ethanol. Cole & Lathe (1953) found that the direct and indirect types of pigment could be separated chromatographically, and Cole, Lathe & Billing (1954) showed that the direct-reacting pigment had two components (pigments I and II). Pigment II is the more polar of the two, and is also the chief pigment of human bile. It occurs, with pigment I, in the serum and urine of patients with obstructive jaundice and hepatitis.

Billing (1954) compared the products of diazotization of bilirubin and of pigment II and found that the azo pigment (pigment B) formed from pigment II was more polar than pigment A formed from bilirubin. The relation between these pigments is shown in Fig. 1. It was suggested that the difference between bilirubin and pigment II could

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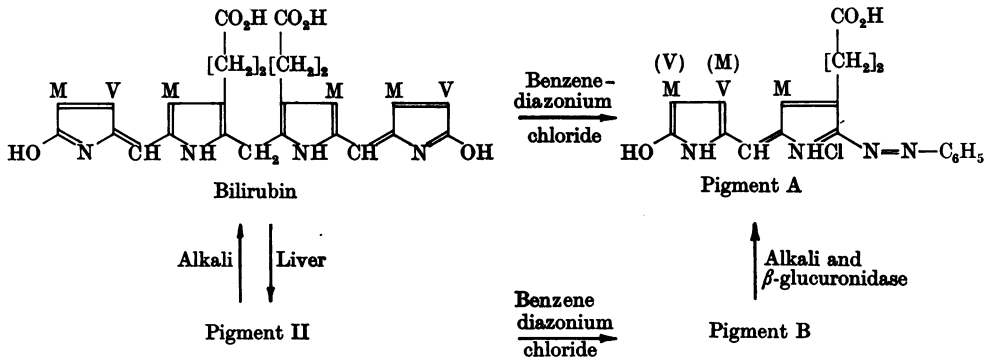

Fig. 1. Relation between bilirubin, pigment II and azo pigments A and B. M, Methyl; V, vinyl.

be predicted from a knowledge of the difference in structure of pigments A and B. The structure of pigment A was established by Fischer & Haberland (1935). Pigment B has now been isolated and shown to be a glucuronide of pigment A. A preliminary report of this work has already appeared (Billing & Lathe, 1956).

Since bilirubin is asymmetrical (Siedel & Fischer, 1933), pigments A and B must each consist of a mixture of two isomers, differing only in the position of the methyl and vinyl side chains. There is some evidence that the isomers have slightly different R_F values on partition chromatograms, but they behave very similarly, and in this work the two isomers of each pigment are discussed as if they were one substance.

EXPERIMENTAL

Analytical

Pigments. Diazotizable bile pigments were estimated by the method of Malloy & Evelyn (1937). Pigments A and B were determined, after dilution in methanol, with a methyl red standard (King, 1951) in a Unicam colorimeter (SP. 300) and an Ilford filter no. 404 (peak transmission 530 mμ.). The standard was equivalent to 4 μg. of bilirubin/ml. and equivalent to 5·12 μg. of pigment A (the benzenediazonium chloride derivative)/ml. At the beginning of the study the nature of pigment B was unknown and it was necessary to express its concentration in terms of pigment A.

Glucuronic acid. Qualitative tests for hexuronic acids were made by the carbazole–H_2SO_4 method of Dische (1947*a*). Glucuronic acid was identified by the mannose–H_2SO_4–thioglycollate method (Dische, 1947*b*), and galacturonic acid was sought by the cysteine–H_2SO_4 method (Dische, 1948).

Quantitative estimations of the glucuronic acid content of bile and of pigment B concentrates were made by the naphtharesorcinol method of Fishman & Green (1955). The amount of glucuronic acid in the purified preparation of pigment B was also determined by reduction of the alkaline-copper reagent of Schaffer & Hartmann (Peters & Van Slyke, 1932). The reagent (0·5 ml.), pigment B solution (0·5 ml., containing 800 μg. of pigment A equivalent) and 5 N-H_2SO_4 (0·2 ml.) were used, according to the directions of Hawk, Oser & Summerson (1947).

Glucuronic acid content of sera before and after preliminary alkaline hydrolysis. A volume of 0·1 N-NaOH (3 ml.) was added to serum (2 ml.) and the solution was left for 30 min. at room temperature; 0·1 N-HCl (3 ml.) and water (10 ml.) were added and mixed, followed by 10% (w/v) sodium tungstate (1 ml.) and 0·66 N-H_2SO_4 (1 ml.). After 10 min. the solution was centrifuged and 4 ml. of protein-free supernatant was used for the determination of total glucuronic acid, according to Fishman & Green (1955). A control tube of 2 ml. of serum and 16 ml. of water was similarly treated, except for alkaline hydrolysis. The bile pigments precipitated with the protein in both tubes.

Spectrophotometry. A Unicam quartz spectrophotometer (SP. 500) was used with 10 mm. quartz cells.

Paper chromatography

Conjugated bile acids. These were identified by the technique of Haslewood & Sjövall (1954). Since the main contaminants of pigment B were glycocholic and glycochenodeoxycholic acids, system G_3, consisting of a mobile phase of *iso*propyl ether–*n*-heptane (3:2, v/v) and a stationary phase of 70% (v/v) aqueous acetic acid, was most useful. Pigment A had an R_F of approx. 0·8, whereas pigment B moved with glycocholic acid (R_F less than 0·1). Pigments A and B stained blue with phosphomolybdic acid but could be identified by visual examination of the papers before spraying.

Amino acids. Pigment B was hydrolysed at 100° in 6 N-HCl for 16 hr. in a sealed tube. After electrolytic desalting and reduction of volume, the hydrolysate was examined by paper chromatography in *sec.*-butanol–formic acid–water (15:3:2, by vol.) as devised by Ahrens & Craig (1952). The pigments moved just behind the solvent front. The amino acids were demonstrated by staining with ninhydrin.

Sugars and uronic acids. Paper chromatography was carried out in *n*-butanol–acetic acid–water (4:1:5, by vol.) and freshly prepared pyridine–ethyl acetate–acetic acid–water (5:5:1:3, by vol.) (Fischer & Dörfel, 1955), respectively. To prepare a glucuronic acid standard, glucuronolactone was dissolved in 0·1 N-NaOH and neutralized. Aniline phthalate (Partridge, 1949) was used to stain reducing substances and a naphtharesorcinol reagent [prepared by mixing equal volumes of 0·2% ethanolic naphtharesorcinol and 2% (w/v) aqueous trichloroacetic acid immediately before use] was employed to show uronic acids and pentoses, which stained blue in the presence of moisture, while ketoses gave a red spot (Partridge, 1948).

Partition chromatography of pigments

Reverse-phase partition columns of 3 g. of silicone-treated kieselguhr in glass tubes of 15 mm. diameter were used. Bilirubin was identified by the $CHCl_3$–CCl_4–methanol (pH 6) solvent system of Cole *et al.* (1954). For the identification and separation of pigments I and II a butanol system at pH 6 was employed (Cole *et al.* 1954).

To separate the azo pigments A and B (prepared from aniline) a system was prepared from *iso*-octanol–methanol–$CHCl_3$–0·1 M acetate buffer, pH 4 (1:10:1:10, by vol.) (Norman, 1954), 2·7 ml. of the lower layer being used as stationary phase; 100–200 μg. of pigment was used. When eluted from this column pigment B was free from conjugated bile acids. When it was desired to elute pigment A as well as B, a system of $CHCl_3$–CCl_4–methanol–0·2 M acetate buffer, pH 4 (25:25:38:12, by vol.) was used; 1·5 ml. of lower layer served as stationary phase.

Countercurrent distribution

Bottles were used for small distributions through 10–15 tubes, the lower layers being moved. The transfers were made with an all-glass syringe and polythene tubing, since exposure to certain metals alters the azo pigments and turns them blue. Distributions of 40–150 tubes were made in the apparatus of Lathe & Ruthven (1951) in which the lower phases are transferred. In order to make 150 transfers in an apparatus of 50 tubes, 63 transfers were first made, after which tubes 11–32, containing most of the pigments, were

placed in tubes 0–21 of the apparatus, respectively, and the distribution was expanded to give a total of 150 transfers. Accordingly, a number of lower phases (which did not contain pigment) were removed from the apparatus without having undergone the full 150 transfers.

Solvent system A was prepared from $CHCl_3$–methanol–0·2M acetate buffer, pH 4 (32:43:25, by vol.). Solvent system B was *n*-butanol–heptane–acetic acid–water (3:7:2:8, by vol.) (Ahrens & Craig, 1952). Solvent system C consisted of *n*-butanol–heptane–acetic acid–water (3:7:1:9, by vol.) (Ahrens & Craig, 1952).

Materials and reagents

Bilirubin and alumina for chromatography were purchased from British Drug Houses Ltd. Hydroxypyrromethenecarbinol was prepared according to the method of Overbeek, Vink & Deenstra (1955*b*). Buffers were prepared according to Cole (1933). Benzenediazonium chloride was prepared according to Cumming, Hopper & Wheeler (1950); the final solution was equivalent to 92 mg. of bilirubin/ml. *p*-Aminobenzoic acid and sulphanilic acid were similarly diazotized.

Mouse-liver β-glucuronidase was prepared according to the directions of Levvy & Marsh (1952). The deposit which was soluble at 31% (w/v) and insoluble at 50% (w/v) $(NH_4)_2SO_4$ was dialysed and kept at 4°. Limpet glucuronidase was supplied by Dr R. D. Bulbrook of the Imperial Cancer Research Fund Laboratory, Royal College of Surgeons, London. It had been prepared by the method of Dodgson & Spencer (1953). Locust-crop juice, which is a rich source of enzyme (Robinson, Smith & Williams, 1953), was obtained at the Anti-Locust Research Centre, Natural History Museum, London. The activity of each preparation was determined at pH 5·2 with 0·01M phenolphthalein β-glucuronide, kindly supplied by Dr C. A. Marsh. The inhibitory effect of boiled 5mM potassium hydrogen saccharate and phenolphthalein β-glucuronide (Levvy, 1952) was demonstrated in these systems.

Preparation of pigment A

Bilirubin (250 mg.) was refluxed in 275 ml. of $CHCl_3$ for 2 hr., filtered and cooled to 5°. Ethanol (80 ml.) was added and then 3·5 ml. of benzenediazonium chloride reagent. After 2 hr., 2 ml. of 2·5N-NaOH was added, with stirring, in order to change the colour from blue to red-blue. After 16 hr. at 5° the solution was washed three times with 100 ml. of water, to remove excess of diazo reagent and acid. The $CHCl_3$ layer was divided into three portions and a brown-red pigment removed by extraction three times with an equal volume of freshly prepared saturated $NaHCO_3$. The $CHCl_3$ layers, which retained most of the red pigment, were combined and extracted with a 50 ml. and a 30 ml. portion of 0·05N-NaOH. The alkaline extracts were combined and brought to pH 3 with 5N-HCl, after which the flocculent pigmented precipitate was separated by centrifuging.

A column for absorption chromatography was prepared by mixing 20 g. of alumina with 20 ml. of 0·01N-NaOH in 85% aqueous methanol. The crude pigment A precipitate (40 mg.), dissolved in 1 ml. of the same solvent and 0·1 ml. of 0·1N-NaOH, was transferred to the top of the column. The column was eluted with 0·01N-NaOH in 85% aqueous methanol and a red band of pigment, poorly defined in front but with a sharp rear edge, was collected in two portions. The faster was shown by partition chromatography to consist almost entirely of pigment A, and the slower also contained a less polar pigment, which was probably a mixture of the methyl esters of pigment A. The first portion of pigment was made acid (approx. pH 5) with dilute acetic acid, extracted into $CHCl_3$ and taken to dryness.

Preparation of pigment B

Human hepatic bile was used as a source of pigment II since gall-bladder bile from human autopsies is usually grossly infected. It was obtained from patients in whom a T-tube had been placed in the common bile duct at operation and was collected daily for a period of 1–20 days after operation. The bile-pigment content varied from 10 to 170 mg./100 ml. of bile. The bile was adjusted to pH 6 with 10N-HCl (approx. 2 ml./l.) with vigorous mechanical stirring.

Twice the theoretical requirement of diazonium reagent was added to the bile. Further addition did not increase the yield of azo pigment, which was about 75% of the estimated amount. The pH was readjusted to 5–6 if necessary, and the bile kept at 4° until processed further.

Concentration of pigment B. Initially 10·6 l. of bile contained diazotizable pigment equivalent to 6·7 g. of pigment A. Coupling yielded 5 g. of pigment B, expressed as pigment A. Ammonium sulphate (500 g./l.) and 20 ml. of *n*-butanol/l. were added, with stirring. After standing at 4° for several hours the lower, pigment-free, aqueous layer was drawn off and discarded. The pigment was concentrated by centrifuging the remainder (1500 *g* for 15 min.), removing the aqueous part, and drying the pad of pigment over $CaCl_2$ for several days, the lumps being broken up periodically. The concentrate (186 g.) was finely ground in a mortar and extracted with 1·3 l. of methanol at 50–60° for 15 min. The mixture was allowed to settle, the supernatant decanted, and the sludge was re-extracted five times with 370 ml. portions of methanol. The residue was discarded. The supernatant fluids were combined, the amount of pigment was estimated, and a 1 ml. portion dried *in vacuo* over $CaCl_2$, and weighed. The remaining solution was reduced *in vacuo* below 40° to a thick syrup; 3·5 g. of crude pigment B (expressed as pigment A) was obtained.

Removal of lipids, pigment A, taurine-conjugated bile acids and brown pigments. This was carried out by a number of

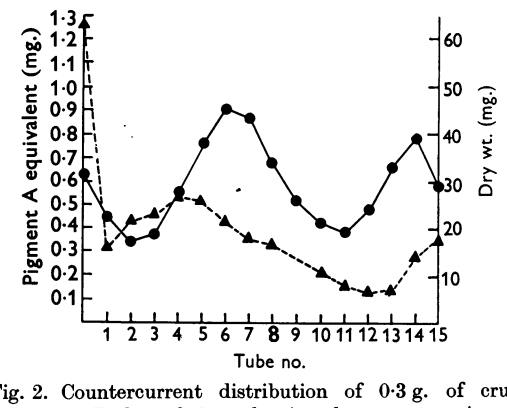

Fig. 2. Countercurrent distribution of 0·3 g. of crude pigment B through 15 tubes in solvent system A, with 225 ml. of upper phase and 15 ml. of lower phase. ●, Pigment (as pigment A); ▲, dry wt.

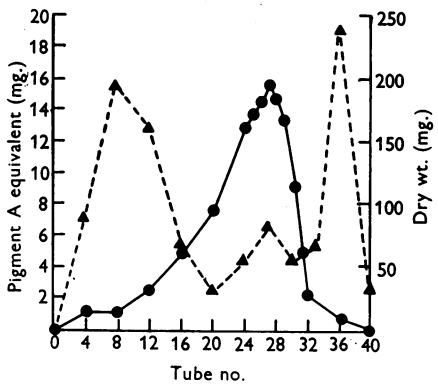

Fig. 3. Countercurrent distribution of 4 g. of pigment B concentrate through 40 tubes in solvent system B, with 24 ml. of upper phase and 36 ml. of lower phase. The distribution was begun with the solute in tubes 0–3. ●, Pigment (as pigment A); ▲, dry wt.

parallel 12–15 tube distributions in solvent system A. After a 15-tube distribution (Fig. 2), and estimation of pigment, tubes 3–10 containing pigment B were combined. Pigment B was moved into the lower phase by adding 40 ml. of aqueous saturated $(NH_4)_2SO_4$/l. of solvent. The separated $CHCl_3$, which contained almost all the pigment, was concentrated to a thick paste under reduced pressure at 40°. At this stage, the yield of pigment B was 1·7 g. (as pigment A).

Separation from glycochenodeoxycholic acid. This was obtained by several distributions in solvent system B (Fig. 3). Analysis showed that glycochenodeoxycholic acid was present in the first 20 tubes, and pigment B, together with glycocholic acid, was distributed in tubes 22–32. Tubes 22–32 were combined, and the pigment was moved into the *n*-butanol–heptane layer by adding 200 g. of $(NH_4)_2SO_4$/l. of solvent mixture. The almost colourless lower layer was discarded and most of the acetic acid was removed from the upper layer by washing four times with 0·25 vol. of half-saturated $(NH_4)_2SO_4$ and finally with 0·25 vol. of water. An equal volume of light petroleum (b.p. 40–60°) and 0·25 vol. of freshly saturated aqueous $NaHCO_3$ solution were added. The mixture was shaken and more powdered $NaHCO_3$ was added until most of the pigment had moved to the lower aqueous phase, and the pH was about 5. The aqueous phase was removed, and the upper layer again shaken with saturated aqueous $NaHCO_3$. If this took up pigment the procedure was repeated. To the combined aqueous layers one-third of the weight of $(NH_4)_2SO_4$ was added. A pigmented butanol layer separated out. This was used to form part of the solvent system for the next distribution. At this stage the yield of pigment B was 1·1 g. (as pigment A).

Separation from glycocholic acid. This was done by two parallel 150-tube distributions in solvent system C (Fig. 4). Most of the glycocholic acid was found in tubes 60–110. The tubes containing pigment B (30–59) were combined and the pigment was concentrated by the technique used after distribution in solvent B. The *n*-butanol residue was dried *in vacuo*. The final yield of pigment B was 0·65 g. (expressed as pigment A).

In the preparation from one batch of bile, a second peak of less-polar pigment was observed in a 150-tube distribution. Chromatographic examination of this material showed that it was probably a complex of pigment A and pigment B. It contained 1 molecule of glucuronic acid to 2 molecules of pigment A. Since it did not appear in subsequent distributions it was assumed to be an artifact and no further examination was made.

Precipitation of pigment B. A portion of the butanol concentrate of purified pigment B, equivalent to 56 mg. of pigment A, was added to 3 ml. of methanol, which dissolved almost all the pigment. A small amount of black deposit was washed with 1 ml. of methanol and then discarded. The methanol extracts were combined and 20 ml. of ethanol was added. A light turbidity was removed by spinning at 1500 *g* for 15 min. The supernatant was then placed in a tube at −12° for 24 hr., when a precipitate was observed. The tube was spun at 1500 *g* for 10 min. and decanted. The precipitate was dried *in vacuo* over $CaCl_2$ at room temperature to a constant weight of 18·3 mg.

Preparation of pigment I from serum

Specimens of serum from cases of obstructive jaundice were extracted with ethanol (Cole *et al.* 1954) and the resulting pigments run on partition columns. When pigment II had been eluted, the portion of the column containing the pigment I band was extracted with ethanol and the combined extracts, from several columns, were analysed for pigment and glucuronic acid.

An aqueous solution of pigment I was prepared by reducing the ethanolic solution to dryness *in vacuo*, and dissolving it in water, or by eluting the pigment I from the column and then extracting the *n*-butanol with light petroleum (b.p. 40–60°). The amounts of direct- and indirect-reacting pigment were then determined.

Effect of β-glucuronidase on pigment B

Pigment B (3 ml., equivalent to 200 μg. of pigment A) and enzyme in buffer, pH 5·2, were incubated for 1 hr. at 37° and then 7 ml. of ethanol was added. The supernatant fluid was taken to dryness *in vacuo*, and the pigmented residue was dissolved in 0·5–1 ml. of the mobile phase for partition chromatography. The bands of pigments B and A were eluted separately and estimated colorimetrically.

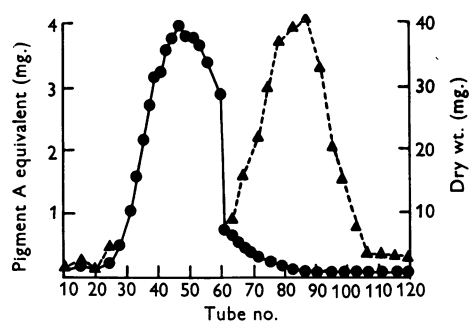

Fig. 4. Countercurrent distribution of 3·3 g. of pigment B concentrate through 150 tubes in solvent system C, with 15 ml. of upper phase and 40 ml. of lower phase. The distribution was begun with the solute in tubes 0–3. ●, Pigment (as pigment A); ▲, dry wt. The dry wt. of the contents of tubes 26–63 was not determined.

Effect of alkali on pigment B

Pigment B (equivalent to 100 μg. of pigment A) was dissolved in 3 ml. of 0·1 N-NH_3 soln. The same amount of pigment B was dissolved in 3 ml. of M phosphate buffer, pH 5, and both solutions were incubated at 37° for 16 hr. The pH was then adjusted to 4 and the solutions were taken to dryness and examined for pigments A and B by partition chromatography. Other samples were similarly treated and, after incubation and electrolytic desalting, the hydrolytic products were examined by paper chromatography for sugars and amino acids.

Effect of alkali on pigment II

Bile (1 vol.) was added to 1·5 vol. of 0·1 N-NaOH at room temperature. At varying intervals from 1 to 120 min., 0·2 ml. portions were removed and added to 4 ml. of 0·2M acetate buffer, pH 4, containing 0·12 ml. of 0·1 N-HCl. A control was similarly treated but without the addition of alkali. Freshly diazotized 0·4% (w/v) sulphanilic acid in 0·25 N-HCl (1·4 ml.) was then added immediately to tests and control, to determine the amount of direct- and indirect-reacting pigment. The nature of the indirect-reacting pigment produced from pigment II was examined by neutralizing the mixture after 7 min., extracting the pigment with $CHCl_3$, and running it on a reverse-phase partition column, alone and with added bilirubin.

RESULTS

In preliminary experiments for determining the most satisfactory type of pigment B, the products formed by coupling pigment II with diazotized sulphanilic acid, *p*-aminobenzoic acid and aniline were examined on partition columns. All were stable and were more polar than the corresponding azo pigments formed from bilirubin. R_F values are given in Table 1. For large-scale isolation of pigment B, aniline was chosen to prepare the diazonium reagent, since the azo pigment produced was the least polar, and thus could be most easily separated from taurine-conjugated bile salts, and other polar interfering substances present in bile.

Properties of pigment B

When examined microscopically pigment B consisted of deeply pigmented spheres of remarkably uniform size, about 10 μ. diameter. In this relatively pure form pigment B was soluble in water at a reaction greater than about pH 4, soluble in methanol, but much less so in ethanol, and almost insoluble in dry *n*-butanol, chloroform, ether and light petroleum. It was stable for several weeks when dried, or at −12° in methanol, although the gradual appearance of a less polar band in partition chromatograms suggested that methylation was taking place. It was stable at pH 2 and at neutrality.

The shape of the absorption curve of pigment B in methanol did not differ appreciably from that of pigment A in the range 220–620 mμ. There were peaks of absorption at 270 and 328 mμ. as well as the main band of absorption at 517 mμ. $E_{525\,m\mu}$. of the purest preparation of pigment B was 0·57 of that calculated for pigment A (methyl red standard).

The addition of 0·1 N-NaOH (approx. pH 12) for a few minutes, or 0·1 N-NH_3 soln. (approx. pH 11) at 37° for 16 hr., changed the solubility of the pigment so that it now precipitated when the pH was adjusted to 5–6. Partition chromatography of the product showed that it moved with an R_F value identical with that of pigment A.

The aqueous products resulting from alkaline treatment of pigment B were examined for amino acids by paper chromatography. The only spot which stained with ninhydrin was faint and moved with an R_F of 0·3, like glycine. Aniline phthalate staining of paper chromatograms showed one brown spot, which moved with an R_F of 0·21, as does glucuronic acid. It stained blue with naphtharesorcinol (as do uronic acids and pentoses). Unhydrolysed pigment B moved with an R_F of about 0·9 in the system used for sugars and there was no other reducing spot.

The uronic acid which was released by alkaline hydrolysis of pigment B had an $R_{glucose}$ value of 0·36, compared with 0·34 for glucuronic acid and 0·29 for galacturonic acid, in pyridine–ethyl acetate–acetic acid. Glucuronic acid and the unknown substance stained brown with aniline phthalate, and galacturonic acid gave a pink spot. The water-soluble product of alkaline hydrolysis gave the specific carbazole–H_2SO_4 test for uronic acids. Galacturonic acid and mannuronic acid were excluded by the

Table 1. *R_F values on silicone-treated kieselguhr columns of azo pigments from bilirubin and pigment II*

Details of the partition systems are in the Experimental section.

Diazonium reagent prepared from	Partition system of *iso*-octanol–methanol–$CHCl_3$–buffer (pH 4)		Partition system of $CHCl_3$–CCl_4–methanol–buffer (pH 4)	
	R_F of pigment A	R_F of pigment B	R_F of pigment A	R_F of pigment B
Sulphanilic acid	1·0	1·0	1·0	1·0
Benzoic acid	0·3	0·8	0·7	1·0
Aniline	<0·1	0·5	0·5	1·0

mannose–thioglycollate–H_2SO_4 test, and galacturonic acid was excluded by the cysteine–H_2SO_4 test, which was negative.

Glucuronic acid content of pigment B

Pigment A gave a negative result in the naphtharesorcinol test.

The molar ratio of pigment A to glucuronic acid, in a sample of precipitated pigment B (which contained 57 % of pigment A by colorimetry), was 1·02 when the naphtharesorcinol method was used, and 0·99 with the reduction method. Calculations from their molecular weights (pigment A, 367; glucuronic acid, 196) showed that 87 % of the precipitated pigment B consisted of pigment A and glucuronic acid.

Effect of β-glucuronidase on pigment B

The β-glucuronidase preparations (from limpet, mouse liver and locust-crop juice) brought about some conversion of pigment B into pigment A, whereas the pigment in the control tube remained unaltered (Table 2).

The specific inhibitors of β-glucuronidase, boiled potassium hydrogen saccharate solution and phenolphthalein β-glucuronide, caused inhibition of the conversion of pigment B into A by limpet and mouse-liver preparations. Previous adjustment of the limpet-enzyme preparation to pH 2·25, which destroys the β-glucuronidase activity of this preparation, also destroyed its ability to change pigment B into pigment A.

The conversion of pigment B into A by locust-crop juice was not, however, inhibited by 5 mM saccharate solution, although this inhibitor was found to be effective in reducing the hydrolysis of phenolphthalein β-glucuronide. The effect of 3 mM phenolphthalein β-glucuronide as a competitive inhibitor was variable; in one experiment 34 % inhibition was observed, but in two other experiments there was no inhibition. Boiled locust-crop juice had no effect on pigment B.

Glucuronic acid content of bile

Eighty-six specimens of fistula bile (none of which was used for the above preparation) were collected from fifteen patients and analysed for direct and indirect pigments and for glucuronic acid. The relation between the content of direct-reacting pigments and of glucuronic acid is shown in Fig. 5. In twenty-six of these specimens (seven from one patient) the molar ratio of glucuronic acid to direct pigment was 2 or more. Forty-three specimens had molar ratios between 1·5 and 2.

Effect of alkali and β-glucuronidase on pigment II

Since pigment B was alkali-labile, the effect of alkali on pigment II was studied by following the change in the amount of direct and indirect pigments after 1 vol. of bile had been added to 1·5 vol. of

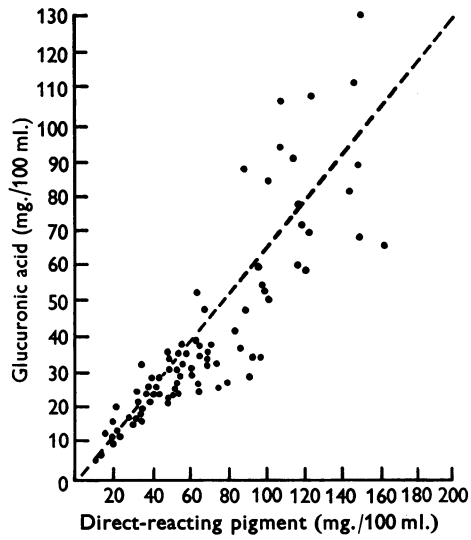

Fig. 5. Content of direct-reacting pigment, and of glucuronic acid, in 86 specimens of human hepatic bile. The broken line represents concentrations having a molar ratio between pigment and glucuronic acid of 2.

Table 2. *Effect of β-glucuronidase preparations on pigment B*

Enzyme preparation	Conversion into pigment A (%)	Inhibition (%)
Limpet	54	—
After acid treatment (pH 2·25)	Nil	100
+5 mM saccharate	6	89
+3 mM phenolphthalein β-glucuronide	23	57
Mouse liver	17	—
+5 mM saccharate	Nil	100
+3 mM phenolphthalein β-glucuronide	6	65
Locust-crop juice	86	—
After heat treatment	Nil	100
+5 mM saccharate	84	2
+3 mM phenolphthalein β-glucuronide	57–86	0–34

0·1 N-NaOH at room temperature (Fig. 6). The direct-reacting pigment was rapidly transformed into approximately an equivalent amount of indirect-reacting pigment. This was identified on a partition column as bilirubin. The breakdown of bilirubin, which is formed in this way, could be explained by the presence of the bile, which had a similar effect on the pure compound under these conditions.

Fig. 6 also shows that a small part of the direct-reacting pigment was alkali-stable, as indicated by the relatively constant amount of direct-reacting pigment present after 30 min. of alkali treatment. The amount of this component varied from 5 to 40 % of the whole in different bile specimens, and was usually 10–15 %. It was not proportionately elevated in those specimens which contained high concentrations of total pigment.

It is possible that another pigment may be present in some specimens of bile, for alkaline treatment of the hepatic bile of six patients during the immediate period after operation revealed a marked drop in the direct-reacting component without a commensurate rise in the indirect one. In three cases the more usual behaviour, represented in Fig. 6, had returned by post-operative days 5–7, but in two cases the unusual behaviour was still present, though reduced in amount, at days 9 and 11. This effect may have been due to a greatly enhanced breakdown of bilirubin.

It was not possible to determine the effect of mouse-liver β-glucuronidase on the pigment of fistula bile, since it was observed that bile markedly inhibited the activity of the enzyme when phenolphthalein β-glucuronide was used as substrate.

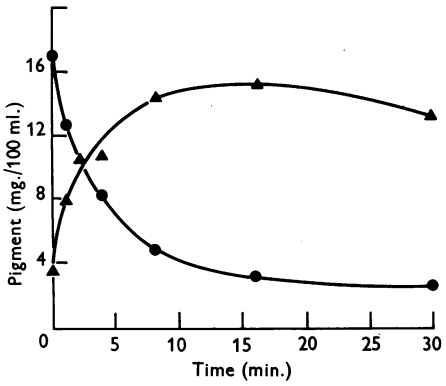

Fig. 6. Change of direct-reacting pigment of hepatic bile into indirect-reacting pigment by 0·06 N-NaOH. At zero time 1 vol. of bile was added to 1·5 vol. of 0·1 N-NaOH. ●, Direct-reacting pigment; ▲, indirect-reacting pigment.

Pigment I

In pigment I which had been separated chromatographically the molar ratio of pigment (expressed as bilirubin) to glucuronic acid was found to vary from 0·8 to 1·2. This variability is probably due to the low concentrations of pigment available for the determinations. Aqueous solutions of pigment I gave a direct reaction in the van den Bergh test. The addition of ethanol to the reaction mixture after 30 min. increased the yield of azo pigment by approx. 40 %.

Alkali-labile glucuronides of serum

The glucuronic acid content of sera from six patients with obstructive jaundice (containing pigments I and II) and three babies with haemolytic disease (whose predominant serum bile pigment was bilirubin) was compared with that of sera from five normal persons. This was done with and without preliminary alkaline hydrolysis of the serum, since without hydrolysis the glucuronic acid component of pigments I and II would be removed because of the affinity of direct-reacting pigments for precipitated plasma proteins. Treatment with alkali increased the apparent glucuronide content of sera from haemolytic patients, and from normal persons, by less than 0·5 mg./100 ml. With sera from patients with obstructive jaundice (containing varying amounts of pigments I and II) the increase ranged from 1·7 to 3·6 mg./100 ml.

DISCUSSION

Coupling of bilirubin and pigment II with diazonium compounds

Fischer & Haberland (1935), from their observations on the dimethyl esters of mesobilirubin III α and XIII α, concluded that the coupling of bilirubin with diazonium compounds involved a preliminary rupture of the methene bridge with the resulting production of pigment A and hydroxypyrromethenecarbinol. Overbeek *et al.* (1955*b*) showed that, in the presence of sufficient coupling agent, the hydroxypyrromethenecarbinol was converted into pigment A and thus that 2 molecules of pigment A are formed from 1 molecule of bilirubin. Since pigment II forms only one pigment in this coupling reaction it can be concluded, by analogy, that 1 molecule of pigment II will form 2 molecules of pigment B. Pigment B has been shown to be a derivative of pigment A and it is, therefore, proposed that the formation of pigment II involves a similar substitution in each half of the bilirubin molecule.

Structure of pigment B

Proof that pigment B was the ester glucuronide of pigment A depended on the observation that under mild alkaline conditions, in which ester glucuronides are labile (Williams, 1947), pigment B yielded pigment A and glucuronic acid. The latter was identified by chromatography and by specific tests. The molar ratio of pigment A to glucuronic acid was approximately 1. β-Glucuronidase preparations from limpet, locust juice and mouse liver also hydrolysed pigment B to pigment A (Table 2). The specificity of this enzyme activity was shown, in the mouse and limpet preparations, by inhibition of the enzymic hydrolysis with boiled saccharate and phenolphthalein β-glucuronide. Conversion of pigment B into pigment A by the limpet preparation was also stopped by prior exposure to pH 2·25; this treatment inhibits β-glucuronidase but not aryl sulphatase activity (Dodgson & Spencer, 1953). With the locust enzyme the lack of inhibition by saccharate was unexplained, as the activity of this enzyme preparation against phenolphthalein β-glucuronide was inhibited by saccharate. With this exception all the evidence suggested that pigment B was an ester glucuronide of pigment A.

The quantitative analysis of the purest pigment B preparation indicated that it was 87 % pigment A glucuronide. The remaining impurity was probably glycocholic acid, since the amount of glycine observed on paper chromatography was consistent with about 15 % contamination with this substance. This was not surprising in view of their similar behaviour in countercurrent distributions (Figs. 3, 4) and in paper chromatography (system G_3 of Haslewood & Sjövall, 1954).

Structure of pigment II

The finding that pigment B was the glucuronide of A suggested that pigment II was bilirubin diglucuronide. A monoglucuronide structure was excluded since coupling of pigment II in small-scale trials yielded only pigment B, and not equal amounts of A and B as a monoglucuronide structure would have required. Under mild alkaline conditions pigment II produced bilirubin, as shown by its chromatographic behaviour and indirect van den Bergh reaction (Fig. 6). The glucuronic acid content of hepatic-bile specimens was usually a little less than that necessary for conjugation with both carboxyl groups of all the direct-reacting pigment (Fig. 5). However, a variable amount of alkali-stable direct-reacting pigment was observed in all specimens (Fig. 6). If this was not a glucuronide, as appears probable, it may account for the frequent occurrence of a minor deficit in the glucuronic acid content of bile.

Theoretically it was conceivable that pigment II was a dipyrrole pigment which combined to form bilirubin. Fischer & Haberland (1935) considered that some dipyrroles could yield bilirubinoid pigments. However, there is no direct evidence that bilirubin is excreted as a dipyrrole compound. Moreover, the production of bilirubin from pigment II by alkaline hydrolysis was strong evidence that the latter was also a tetrapyrrole pigment, and that at the central methene group it had the same structure as bilirubin.

The yield of pigment B in the large-scale preparation was less than 5 % of that contained in the original bile. This is due mainly to such factors as incomplete conjugation, relatively poor extraction with methanol from the dry extract, the degradation of pigment B to A during the various procedures (most noticeable in Fig. 2), and the discarding of countercurrent tubes containing some pigment B, together with large amounts of impurities. The possibility that larger amounts of some other azo pigment similar to B was removed during the preparation has had to be considered. However, partition chromatograms of the original diazotized biles showed only one type of pigment B and, at all stages in the preparation, analyses of specimens of pigment B obtained from peak tubes of countercurrent distributions indicated a molar ratio of approximately 1 for pigment to glucuronic acid. There was no evidence that the original pigment B contained an appreciable amount of non-ester glucuronide component, as might have arisen from the alkali-stable direct pigment shown in Fig. 6. Thus the low yield of pigment B is not inconsistent with bilirubin diglucuronide being the main pigment of human bile.

It would have been more satisfactory if the postulated structure for pigment II could have been supported by the results of direct glucuronic acid analysis of a band of pigment II from a partition column, or in a countercurrent distribution. However, it has not yet been possible to develop a satisfactory partition column for this purpose, and the analysis of small countercurrent distributions of bile gave extremely variable results. It was uncertain how much of this was due to the breakdown of pigment II during the distribution, or to irregular behaviour as a result of complex formation with bile salts. Attempts to show that liver β-glucuronidase changed the direct-reacting pigment of bile to an indirect one were also unsuccessful. This was attributed to the presence of a β-glucuronidase inhibitor in bile, as it was found that the addition of bile inhibited the hydrolysis of phenolphthalein β-glucuronide by mouse-liver enzyme. The occurrence of natural inhibitors of β-glucuronidase in tissue extracts has previously been noted (Levvy, 1953).

Although further work is necessary to clarify some of these aspects, the evidence indicates that bilirubin is excreted by the liver as a diglucuronide. The alkali lability suggests that the linkage is of an ester type, the propionic acid side chains of bilirubin linking with the $C_{(1)}$ hydroxyl of glucuronic acid. A number of aromatic acids and aliphatic branched acids are excreted in the urine as glucuronides of this type (Kamil, Smith & Williams, 1953), nicotinic acid (van Eys, Touster & Darby, 1955), aspartic and glutamic acids (Pollack & Eades, 1954). Glucuronides of thyroxine (Taurog, Briggs & Chaikoff, 1952) and of oestriol (Kinsella, Francis, Thayer & Doisy, 1956) probably occur in bile.

The same conclusion has been reached independently by Talafant (1956*a*), who showed that, in paper electrophoresis of bile, pigment II was constantly associated with glucuronic acid in a molar ratio 1:2. β-Glucuronidase preparations yielded bilirubin. The lead salt of bilirubin diglucuronide has also been prepared (Talafant, 1956*b*). Schmid (1956) has also presented paper-chromatographic and enzymic evidence that pigment B is the glucuronide of pigment A, but has suggested that the linkage is of the glycosidic type.

Pigment I

Pigment I yields both pigment A and B in the diazo reaction (Billing, 1954). This is consistent with a monoglucuronide structure. In determinations of the molar ratio of glucuronic acid to pigment I a value of about 1 was found. The possibility that pigment I is a complex of bilirubin and pigment II has had to be considered in view of our previous report that pigment I could be converted into bilirubin and pigment II by warming and evaporating its solutions and rechromatographing them. The material available for study has been limited, and some but not all of the specimens have shown this transformation. The present evidence therefore favours a monoglucuronide structure.

Although pigment I couples directly in the van den Bergh test, it should yield some hydroxypyrromethenecarbinol. We have found that the coupling of hydroxypyrromethenecarbinol with diazonium compounds requires the presence of ethanol for its conversion into pigment A. Accordingly, it was to be expected that the yield of azo pigments formed would be increased by the addition of ethanol. In a few trials the increase amounted to about 40%. This would also result from mixtures of bilirubin and pigment II.

Differences between bilirubin and pigment II

A number of differences between direct- and indirect-reacting pigments have been noted in the past, especially with regard to solubility, occurrence in urine, dialysability, affinity for serum protein and protein precipitates, ease of oxidation, affinity for brain tissue and association with different types of jaundice. Some of these differences can be explained on the basis of conjugation with glucuronic acid.

A difference in solubility of direct- and indirect-reacting pigments has been known for some time. Bilirubin is lipid-soluble (approx. 0·1% in chloroform), and its solubility in water at neutrality is negligible (0·0001%) according to Overbeek, Vink & Deenstra (1955*a*). In contrast, pigment II is insoluble in chloroform and soluble in water. It occurs in bile in concentrations up to 0·4%. It is well known that conjugation with glucuronic acid greatly increases the water solubility of many substances.

The insolubility of bilirubin in water at neutrality probably explains why bile pigments do not appear in the urine in those conditions in which bilirubin is the chief serum pigment (acholuric jaundice, haemolytic jaundice). In obstructive jaundice and hepatitis, however, pigments I and II occur in the serum. These pigments are water soluble and can therefore be excreted in the urine. Hitherto the occurrence of direct-reacting pigments in the urine has been attributed to their greater dialysability. In our experience both types will dialyse slowly if the reaction is such that they are soluble. Under physiological conditions the decisive factor determining the dialysability of direct pigments and the non-dialysability of bilirubin is the solubility of the former and the insolubility of the latter in aqueous solution.

It has been suggested that 'indirect' and 'direct' bilirubin are attached to different plasma proteins, and that this explains their difference in behaviour. Gray & Kekwick (1948) found that both were attached to albumin. A report by Najjar (1952) based on salting-out techniques, and indicating that direct-reacting pigment has an affinity for albumin, and indirect-reacting pigment for globulin, was not confirmed by Klatskin & Bungards (1956). Nevertheless, denatured protein, as obtained by ethanol precipitation, has a greater affinity for direct-reacting pigments than for bilirubin. This may also be partly a question of solubility, but some other unknown factor is probably involved.

A striking difference between the two types of pigment is shown by a greater ease of oxidation of direct-reacting pigments in body fluids. The greater stability of bilirubin may be partly due to its association with lipids which exert an antioxidant action. The lipids in bile seem to protect pigment II in the same way, since removal of lipid with chloroform is followed by rapid oxidation. The affinity of bilirubin for brain tissue (Claireaux, Cole & Lathe, 1953), and its toxic effect, probably also depend on its lipophilic characteristic, as the water-soluble direct-reacting pigments do not stain brain tissue,

and are non-toxic. In this respect the behaviour of bilirubin is similar to that of morphine, which is also lipid-soluble (and water-insoluble) and is excreted as a water-soluble glucuronide, which has no effect on the brain (Woods, 1954).

The distinction between direct and indirect reactions in the van den Bergh test is probably also determined by the different solubility of the pigments, as ethanol has to be added only to the pigment which is insoluble in water (at the acid reaction of the test). However, bilirubin will couple directly in the absence of ethanol if the reaction is alkaline (Gedigk & Gries, 1952), since bilirubin then exists as a soluble salt. The importance of solubility is also born out by the recent work of Jirsa, Večerek & Ledvina (1956), who prepared the mono- and ditaurine conjugates of bilirubin. These synthetic pigments, which are also soluble at an acid reaction, behave like the natural bilirubin conjugates (pigments I and II) in partition chromatography and electrophoresis, in regard to ease of oxidation, and in the van den Bergh reaction.

The association of direct-reacting serum pigments with obstructive rather than haemolytic jaundice suggests that this pigment can be formed only in the liver. *In vitro* studies indicate that only liver tissue has a high capacity to form glucuronides (Karunairatnam, Kerr & Levvy, 1949). The kidney was found to have only one-quarter of this activity. Bollman & Mendez (1955) and Bollman (1956) noted that in the hepatectomized dog there was a slow conversion of bilirubin into pigment I. As pigment II of dog bile is also bilirubin diglucuronide (our unpublished observations) this suggests that *in vivo* the capacity to conjugate with glucuronic acid is not entirely confined to the liver, and perhaps the formation of the monoglucuronide may be effected by the kidney.

SUMMARY

1. Pigment II, the direct-reacting pigment of human bile, when coupled with benzenediazonium chloride, forms azo pigment B, whereas bilirubin forms azo pigment A. A comparison of the properties of these two azo pigments indicated that pigment B is the ester glucuronide of pigment A.

2. It has been shown that bilirubin is excreted in human bile as an ester diglucuronide.

3. The other direct-reacting bile pigment, pigment I, which occurs in serum and urine together with pigment II, is probably an ester monoglucuronide of bilirubin.

4. The difference in the type of van den Bergh reaction, and in the physiological behaviour of bilirubin and pigments I and II, can be accounted for by the fact that bilirubin is lipid-soluble, whereas the bilirubin glucuronides are water-soluble.

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