Biochem. J. (1970) **119**, 387–394
Printed in Great Britain

Bilirubin Conjugates of Human Bile

ISOLATION OF PHENYLAZO DERIVATIVES OF BILE BILIRUBIN

BY CLIVE C. KUENZLE*
Institute of Organic Chemistry, University of Zürich, 8001 *Zürich, Switzerland*

(*Received* 19 *January* 1970)

A method is presented that allows the isolation of eight different phenylazo derivatives of bile bilirubin. In step I of the isolation procedure, three bilirubin fractions (bilirubin fractions 1, 2 and 3) from human hepatic bile are separated by reverse-phase partition chromatography on silicone-treated Celite with the use of a solvent system prepared from butan-1-ol and 5mM-phosphate buffer, pH 6.0. Azo coupling is then performed with diazotized aniline. The three azo pigment mixtures are subjected to step II, in which the above chromatography system is used again. With each azo pigment mixture this step brings about the separation of a non-polar and a polar azo pigment fraction (azo 1A and azo 1B, azo 2A and azo 2B, and azo 3A and azo 3B from bilirubin fractions 1, 2 and 3 respectively). Approximately equal amounts of non-polar and polar pigments are obtained from bilirubin fractions 1 and 2, whereas bilirubin fraction 3 yields azo 3B almost exclusively. In step IIIA the non-polar azo pigment fractions are fractionated further by adsorption chromatography on anhydrous sodium sulphate with the use of chloroform followed by a gradient of ethyl acetate in chloroform. Three azo pigments are thus obtained from both azo 2A (azo $2A_1$, azo $2A_2$ and azo $2A_3$) and azo 3A (azo $3A_1$, azo $3A_2$ and azo $3A_3$). The 2A pigments occur in approximately the following proportions: azo $2A_1$, 90%; azo $2A_2$, 10%; azo $2A_3$, traces. The pigments are purified by crystallization, except for the A_3 pigments, which are probably degradation products arising from the corresponding A_2 pigments. In step IIIB the polar azo pigment fractions are subjected to reverse-phase partition chromatography on silicone-treated Celite with the use of a solvent system prepared from octan-1-ol–di-isopropyl ether–ethyl acetate–methanol–0.2M-acetic acid (1:2:2:3:4, by vol.). Azo pigment fractions 2B and 3B each yield six azo pigments (azo $2B_1$ to azo $2B_6$ and azo $3B_1$ to azo $3B_6$ respectively) together with small amounts of products of hydrolysis (azo $2A_B$ and azo $3A_B$). Only one azo B pigment is obtained from bilirubin fraction 1, and this azo pigment is probably of the B_2 type. The yields of the azo 3B pigments suggest that these pigments are present in approximately the following proportions: azo $3B_1$, 0–0.4%; azo $3B_2$, traces; azo $3B_3$, traces; azo $3B_4$, 10%; azo $3B_5$, 50%; azo $3B_6$, 40%. Azo pigments $2B_1$ to $2B_6$ are estimated to occur in similar proportions. Since pairs of correspondingly numbered azo pigments from bilirubin fractions 1, 2 and 3 do not separate on rechromatography together (e.g. azo $2A_1$ co-chromatographs with azo $3A_1$, and azo $2B_6$ co-chromatographs with azo $3B_6$), it is concluded that such pigments are chemically identical. The structures of the isolated phenylazo derivatives are discussed in an accompanying paper (Kuenzle 1970*c*).

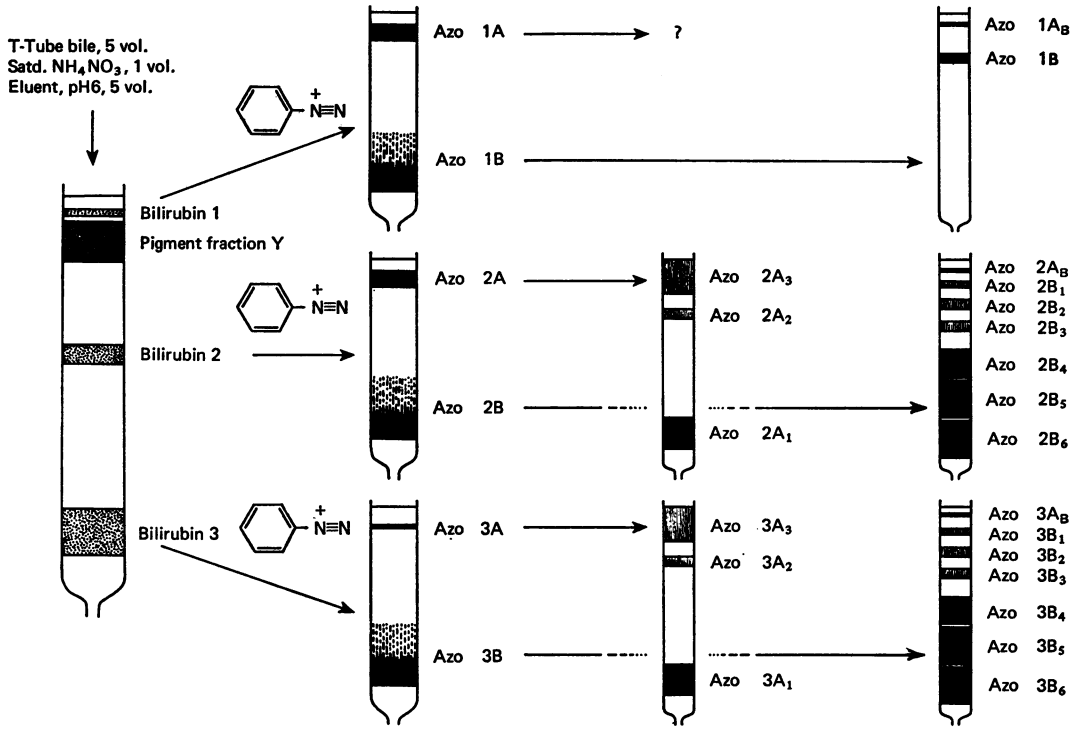
Bilirubin IXα (Fischer & Plieninger, 1942), the linear tetrapyrrole arising from metabolic breakdown of haemoglobin, myoglobin and other porphyrin compounds, is converted into water-soluble derivatives by the action of liver enzymes. This process, termed conjugation, occurs not only with bilirubin but also with many other metabolites and with drugs. Conjugation allows these otherwise water-insoluble compounds to be excreted in the bile and urine. Inadequate or impaired conjugation causes accumulation of the parent compounds in the blood, as is the case with bilirubin in certain forms of jaundice. Non-specific or enzymic hydrolysis of the bilirubin derivatives of bile may

* Present address: Institute of Pharmacology, University of Zürich, Gloriastrasse 32, 8006 Zürich, Switzerland.

play an important role in the formation of gallstones. Knowledge of the naturally occurring bilirubin derivatives is therefore of considerable interest.

Bilirubin was reported to be excreted in human bile as an alkali-labile glucuronide (Talafant, 1956; Billing, Cole & Lathe, 1957; Schmid, 1957) giving the direct van den Bergh reaction (van den Bergh & Muller, 1916). An ester glucuronidic linkage involving the propionic acid side chains of bilirubin was proposed (Billing *et al.* 1957) and was confirmed subsequently by Schachter (1957). However, some of the direct-reacting bilirubin of bile was found to be resistant to alkaline hydrolysis (Billing *et al.* 1957; Isselbacher & McCarthy, 1959). This finding suggested the occurrence of bilirubin conjugates other than an acyl glucuronide. Ethereal and *N*-glucuronides (Gregory & Watson, 1962*b*), sulphates (Isselbacher & McCarthy, 1959; Gregory & Watson, 1962*b*; Schoenfield, Bollman & Hoffman, 1962), phosphates (Tenhunen, 1965) as well as methyl (Isselbacher & McCarthy, 1959), glycine (Jirsa & Večerek, 1958; Jirsa, Ledvina & Večerek, 1958; Isselbacher & McCarthy, 1959) and taurine (Jirsa & Večerek, 1958; Jirsa *et al.* 1958) conjugates of bilirubin have been proposed as possible structures. Bilirubin sulphate was shown to exist in rats (Isselbacher & McCarthy, 1959; Gregory & Watson, 1962*b*; Schoenfield *et al.* 1962), but its occurrence in humans is still controversial (Isselbacher & McCarthy, 1959; Gregory & Watson, 1962*b*; Vegas, 1963; Tenhunen, 1965; Weber & Schalm, 1965; Noir, Groszman & DeWalz, 1966). None of the other proposed types of bilirubin conjugates have been isolated from biological samples in a form sufficiently pure to permit conclusive identification.

To settle the question of unidentified bilirubin compounds of human bile, I undertook to isolate the phenylazo derivatives of bilirubin (for a discussion of the coupling reaction of bilirubinoid compounds see Kuenzle, 1970*b*) and its conjugates in sufficient quantities for structural analysis. In the first paper of this series I propose to show that several bilirubin conjugates occur in human bile. The second paper (Kuenzle, 1970*b*) deals with the



Scheme 1. Diagrammatic representation of the fractionation procedure used for the isolation of the phenylazo derivatives of bile bilirubin. For details see the Experimental and Results section.

spectra of model compounds, which were to help in the structural analysis of the isolated phenylazo derivatives of bilirubin conjugates. Finally, the third paper (Kuenzle, 1970c) gives an account of the elucidation of the structure of the isolated compounds.

A preliminary account of the present work has appeared (Kuenzle, 1970*a*).

EXPERIMENTAL AND RESULTS

A diagrammatic representation of the overall isolation procedure is given in Scheme 1.

Human hepatic bile was obtained from patients on whom cholecystectomy had been performed for removal of gallstones. A T-tube had been placed in the common bile duct at operation. The bile from the T-tube was collected for several days after operation, and was sampled in plastic bags that were cooled with ice and protected from light. Samples (0.5–1 litre) were frozen and kept at −20°C in the dark. After careful thawing, bile samples from several patients were pooled and were rapidly filtered through cloth. The filtrate was divided into portions and was stored in plastic bottles at −20°C in the dark until processed further.

Quantitative analysis of bilirubin fractions

As a preliminary test, the three bilirubin fractions of bile (Kuenzle, Sommerhalder, Rüttner & Maier, 1966*a*; Kuenzle, Maier & Rüttner, 1966*b*) were measured in bile pool I. This pool was later used to isolate the phenylazo derivatives of the individual bile bilirubin fractions 2 and 3 (see below). Separation of the three bilirubin fractions from 2.0ml of bile was effected by the chromatographic procedure described below (see step I of the isolation procedure) by using a column (5.3cm × 22cm) prepared from 150g of silicone-treated Celite. The eluates of bilirubin fractions 2 and 3 were collected from the outlet of the chromatography tube, and azo coupling was performed by the addition of 1.0 and 4.0ml respectively of a freshly prepared solution of *p*-diazobenzenesulphonic acid (Kuenzle *et al.* 1966*b*). After 30min the reaction mixtures were saturated with solid $(NH_4)_2SO_4$. The red butanolic layer that separated out was collected, filtered and evaporated to dryness (throughout this work evaporation of solvents was performed in a rotary evaporator unless otherwise stated). The portion of the column that contained bilirubin fraction 1 was cut out and was suspended in approx. 20ml of the mobile phase of the above chromatographic solvent system. To the suspension was added 0.4ml of *p*-diazobenzenesulphonic acid. After 30min the reaction mixture was filtered by suction, and the filtrate was extracted, filtered again and evaporated to dryness as described above. The residues of the coupled bilirubin fractions 1, 2 and 3 were dissolved in methanolic 1M-H_2SO_4, filtered into graduated flasks and made up to 10, 100 and 500ml respectively. Colorimetry was performed in quartz cells of 1cm light-path at 570nm with the use of a Unicam SP.500 spectrophotometer. Concentrations of bilirubin were calculated from a millimolar extinction coefficient of 84.9 (Fog, 1964), and were expressed as mg of unconjugated bilirubin/100ml of bile. [Since it has been shown that the optical spectra and the extinction coefficients of the phenylazo derivatives of bilirubin and of bilirubin dimethyl ester are identical (Kuenzle, 1970*b*), and since virtually all of the bilirubin conjugates present in bile are esters of bilirubin (Kuenzle, 1970*c*), it seemed reasonable to employ the same millimolar extinction coefficient for the estimation of the azo derivatives of both bilirubin and the bilirubin conjugates.] The following values were obtained: bilirubin fraction 1, 2.0mg/100ml (1.6% of the total bile bilirubin); bilirubin fraction 2, 17.9mg/100ml (14.2% of the total); bilirubin fraction 3, 106.6mg/100ml (84.2% of the total).

Isolation of the phenylazo derivatives of the individual bile bilirubin fractions 2 and 3

Step I. Ten 330ml portions of filtered bile (bile pool I) were subjected to fractionation. This was carried out by the reverse-phase partition chromatography described below, which was essentially a macro modification of the method used by Kuenzle *et al.* (1966*a*) to separate the three bilirubin fractions of bile. A solvent system was prepared from butan-1-ol and 5mM-sodium–potassium phosphate buffer, pH6.0. Celite was treated with dichlorodimethylsilane and was washed with methanol–aq. NH_3 (sp.gr. 0.88) (2000:1, v/v). Columns (20cm × 120cm) were prepared from 12.6kg of dry silicone-treated Celite, 6.3 litres of the organic layer and an excess of the aqueous layer (mobile phase) of the above solvent system. One column was used for the fraction of each bile portion. The bile was thawed immediately before chromatography and was mixed with 0.2vol. of saturated NH_4NO_3 and 1vol. of mobile phase. The resulting solution was applied to the chromatography column. Mobile phase was allowed to flow through the column at a rate of 1.5 litres/h at 20°C in the dark. The three bilirubin fractions separated within a few hours. In addition, a brownish-yellow zone appeared just below bilirubin fraction 1. The former, termed pigment fraction Y, gave no reaction with diazonium salts (pigment fraction Y was missing from some bile specimens). Bilirubin fraction 3 emerged from the column after approx. 18h, and elution was usually complete within another 6h. Azo coupling in the eluate was immediately performed by the addition of 10ml of a chilled solution of phenyldiazonium chloride freshly prepared from 0.4g of $NaNO_2$ in 5.0ml of water, and 0.5ml of aniline (freshly distilled) in 9.0ml of 1.5M-HCl. The reaction mixture was allowed to stand at 20°C for 30min, during which time it became red–brown. Solid NaCl (1:10, w/v) was added with stirring, and the mixture was left to stand at 0°C until a dark-red butanol layer had completely separated out. The butanol layer was washed once with 4vol. of 10% (w/v) NaCl, and was evaporated to dryness at 25°C by azeotropic distillation with water. The residues from the full series of chromatographic runs were extracted with methanol and were filtered to remove excess of NaCl. The filtrates were combined and were evaporated to dryness at 25°C. The resulting crude azo pigment mixture, termed azo 3, was stored at −20°C in the dark until processed further.

To elute bilirubin fraction 2, the mobile phase was allowed to flow through the column at the original rate

for another 12–16 h (usually overnight). Suction was then applied to the column until bilirubin fraction 2 was completely eluted. Azo coupling was performed with 2 ml of phenyldiazonium chloride solution, and the reaction mixture was treated as described above. The resulting crude azo pigment mixture was termed azo 2.

Step II. Azo 2 and azo 3 (from step I) were subjected to reverse-phase partition chromatography with the use of the above system (see step I).

Azo 3 was dissolved in the minimum volume of the aqueous layer (mobile phase) of the solvent system, pH 6.0, used in step I, and was applied to one column (20 cm × 80 cm) prepared from 8.4 kg of silicone-treated Celite, 4.2 litres of the organic layer and an excess of the aqueous layer of the above solvent system. The mobile phase was allowed to flow through the column at a rate of 1.8 litres/h at 20°C. After approx. 6 h three bands appeared on the column. The front zone, consisting of green and orange pigments, was discarded. The bulk of the red azo pigments, termed azo 3B, moved slowly through the column in a broad band. It emerged from the column after 30 h and was collected for 36 h. A minor azo pigment fraction, termed azo 3A, was located in a narrow zone near the top of the column. Excess of solvent was removed from the column by suction, and the portion containing azo 3A was cut out, eluted with methanol and filtered by suction. The filtrate was evaporated to dryness at 25°C. The residue was dissolved in a mixture of water and ethyl acetate, and the aqueous layer was extracted exhaustively with ethyl acetate. The combined extracts were washed four times with water and evaporated to dryness. The residue was dried further in a high vacuum. It was redissolved in chloroform, filtered and again evaporated to dryness, yielding a residue of azo 3A.

The eluate containing the fraction azo 3B was extracted exhaustively with butan-1-ol at 0°C, and the combined extracts were washed once with 0.2 M-acetic acid. The butanol layer was evaporated to dryness at 25°C by azeotropic distillation with water. The residue of azo 3B thus obtained was dried further in a high vacuum.

Azo 2 was treated in essentially the same manner as azo 3. However, some minor modifications of the procedure were necessary. Dissolution of the azo pigment mixture was effected by vigorous shaking with mobile phase for 1 h. The resulting oily emulsion was applied to one column (12 cm × 45 cm) prepared from 1.6 kg of silicone-treated Celite and was eluted with mobile phase at a rate of 550 ml/h. A brown oil that remained on top of the column was discarded; so was the front zone consisting of green and orange pigments. The two azo pigment fractions, termed azo 2A (non-polar) and azo 2B (polar), were present in approximately equal amounts, in contrast with the ratio observed with azo 3A and azo 3B. The former were eluted and processed as described for azo 3A and azo 3B respectively.

Step IIIA. Azo 2A and azo 3A (from step II) were fractionated further by adsorption chromatography.

Azo 2A was dissolved in 10 ml of chloroform and was applied to one column (3.1 cm × 33 cm) prepared with anhydrous Na_2SO_4. Elution with chloroform gave three azo pigment fractions. The bulk of the pigments, azo $2A_1$, migrated with the solvent front, and were completely eluted with chloroform. The second fraction, termed azo $2A_2$, was then eluted with a gradient of ethyl acetate in chloroform. After prolonged elution with pure ethyl acetate, the faintly coloured third fraction, termed azo $2A_3$, was present in a broad zone. The latter covered the upper two-thirds of the column but failed to subdivide. It was eluted with methanol–acetic acid (10:1, v/v), but the yield was too low for further processing. The eluates containing the fractions azo $2A_1$ and azo $2A_2$ were evaporated to dryness at 25°C. Crystallization from hot methanol and hot methanol–water (3:1, v/v) respectively yielded the pure compounds azo $2A_1$ and azo $2A_2$. Yields are given in Table 1.

Azo 3A was treated as described for azo 2A, but a

Table 1. *Azo pigment yields from three bile pools*

Running no. of bile pool ...	I		II	III	
Vol. of bile processed (ml) ...	3300		6000	6000	Approx. average abundance of azo pigments (% of total azo A and azo B pigments respectively)‡
No. of patients from whom bile pool was obtained	3		9	5	
Bilirubin fraction	2	3	2+3	2+3	
Yields of azo A pigments (mg)					
Azo A_1	13.5	0.3	31.5	Not isolated	90
Azo A_2	3.3	Traces	1.7	Not isolated	10
Azo A_3	Traces	Traces	Traces	Not isolated	Traces
Total	16.8	0.3	33.2	—	100
Yields of azo B pigments (mg)					
Azo B_1	0.3*	1.5	0	0	0–0.4
Azo B_2	0.9*	0.2	1.5	Traces	Traces
Azo B_3	0.7*	0.3	1.6	Traces	Traces
Azo B_4	1.1†	38.7	58.0	35.3	10
Azo B_5	2.4†	199.0	271.6	171.5	50
Azo B_6	2.5†	138.6	215.1	153.8	40
Total	—	378.3	547.8	360.6	100

* Preparations contaminated with large amounts of silica.

† Low yields due to losses caused by rechromatography.

‡ Yields of the azo 2B pigments from bile pool I were not used to calculate the average relative abundances.

smaller column (2.6cm × 26cm) was used. Three azo pigment fractions (azo $3A_1$, azo $3A_2$ and azo $3A_3$) appeared on the column, the chromatographic pattern being similar to the one observed with azo 2A. However, yields of all three azo pigments were far lower than expected, since the main portion of the mixture applied to the column proved to consist of a brown oil that moved just ahead of azo $3A_1$. The yield of impure azo $3A_1$ was 0.3mg, and crystallization was not achieved. The isolation of azo $3A_2$ and azo $3A_3$ was not attempted.

Step IIIB. Azo 2B and azo 3B (from step II) were fractionated further by reverse-phase partition chromatography on silicone-treated Celite. A solvent system, pH3.4, was prepared from octan-1-ol–di-isopropyl ether–ethyl acetate–methanol–0.2M-acetic acid (1:2:2:3:4, by vol.).

Azo 2B was dissolved in 40ml of the aqueous layer (mobile phase) of the above solvent system by vigorous shaking for 20h. The resulting solution was applied to one column (5.2cm × 95cm) prepared from 680g of silicone-treated Celite, 340ml of the organic layer and an excess of the aqueous layer of the same solvent system. The mobile phase was allowed to flow through the column at a rate of 120ml/h at 20°C. After 24h seven azo pigment fractions were clearly separated. These were numbered from top to bottom, and were designated azo $2A_B$, azo $2B_1$, azo $2B_2$, azo $2B_3$, azo $2B_4$, azo $2B_5$ and azo $2B_6$. The solvent was allowed to drain off and was completely removed from the column by suction. The chromatography tube was broken and the fractions were cut out, eluted with methanol and filtered by suction. The filtrates were concentrated at 25°C to yield octanol solutions of the azo pigments.

The solutions of the fractions azo $2A_B$, azo $2B_1$, azo $2B_2$ and azo $2B_3$ were evaporated to dryness at 0.001mmHg at 35°C.

The residue of azo $2A_B$ was dissolved in chloroform and subjected to adsorption chromatography on a column (1.8cm × 13cm) of anhydrous Na_2SO_4 with the use of chloroform as the eluent (see step IIIA). Minute amounts of an azo pigment that moved with the solvent front were obtained.

Pigments azo $2B_1$ to azo $2B_3$ were each dissolved by shaking with 20ml of butan-1-ol and 60ml of 0.2M-acetic acid, and the red butanol layer was evaporated to dryness at 25°C by azeotropic distillation with water. The residue was redissolved in 5ml of methanol and filtered. To the filtrate was added with shaking 3vol. of ethyl acetate followed by 3vol. of hexane, and the solution was slowly concentrated at 20°C to precipitate the pigment partially. To the suspension was added dropwise with shaking 10vol. of dry ether and the precipitated pigment was collected by centrifugation. The precipitate was washed once with dry ether, centrifuged, collected and dried in a high vacuum to yield the compounds azo $2B_1$, azo $2B_2$ and azo $2B_3$ respectively. Yields are given in Table 1.

The octanolic solutions of the fractions azo $2B_4$ to azo $2B_6$ were concentrated at 0.001mmHg at 35°C for 1h. To each concentrate was added dropwise with shaking 2vol. of dry ether. The resulting precipitates were collected by centrifugation, washed with dry ether and dried in a high vacuum. To ensure chromatographic purity of the fractions azo $2B_4$ to azo $2B_6$, each precipitate was rechromatographed with the above system. However, not only were the fractions found to be chromatographically pure, but it also became evident that a loss of approx. 75% of substance occurred during rechromatography. Therefore rechromatography was omitted in all later experiments. The fractions from the second chromatographic run were eluted and concentrated as described above. The pigments were precipitated from the octanolic concentrates by the dropwise addition of 2vol. of dry ether and were left to stand at −20°C overnight. The precipitates were collected by centrifugation at 0°C, washed three times with dry ether and dried in a high vacuum. The pigments were finely powdered and dissolved in a minimum volume of methanol–acetic acid (10:1, v/v). The filtered solutions were concentrated to a small volume at 25°C. To each concentrate was added dropwise with shaking 1vol. of ethyl acetate followed by 1vol. of hexane. The resulting turbid solutions were slowly concentrated at 20°C to precipitate the pigments partially. Further precipitation was effected by the dropwise addition of 0.5vol. of dry ether. After standing at −20°C overnight, the precipitates were collected by centrifugation at 0°C, washed three times with dry ether and dried in a high vacuum. More pigments were obtained from the mother liquors by further concentration followed by the above precipitation procedure. The total yields of the compounds azo $2B_4$, azo $2B_5$ and azo $2B_6$ are given in Table 1.

Azo 3B was treated in essentially the same manner as azo 2B. Azo 3B was dissolved in 400ml of mobile phase, pH3.4, and was applied to one column (9.4cm × 120cm) prepared from 2.8kg of silicone-treated Celite. Chromatography was carried out for 66h at a flow rate of 220ml/h. Again seven azo pigment fractions appeared on the column. These were designated azo $3A_B$ and azo $3B_1$ to azo $3B_6$. Further processing of these fractions was effected as described above, but rechromatography was omitted. The yields of the compounds azo $3B_1$ to azo $3B_6$ are given in Table 1.

Isolation of the phenylazo derivatives of the combined bile bilirubin fractions 2 and 3

Bile pools II and III were processed independently in essentially the same manner as described above, but much larger amounts of bile were applied to only one column in step I. No separation of bilirubin fractions 2 and 3 was thus achieved, and the two fractions were eluted together.

Step I. Silicone-treated Celite (12.6kg) was treated in the usual manner with the two layers of the solvent system, pH6.0. Approximately five-sixths of the resulting slurry was packed into a column (20cm × 100cm). The residual portion of the slurry was added to a solution prepared from 6 litres of filtered bile, 1.2 litres of saturated NH_4NO_3 and 6 litres of the eluent. The suspension was poured on to the column and was stirred until the applied bile had completely entered the column bed. On elution with mobile phase, bilirubin fractions 2 and 3 moved together in a single broad zone, but were separated from bilirubin fraction 1 and pigment fraction Y. Azo coupling of the combined bilirubin fractions 2 and 3 was performed with 80ml of phenyldiazonium chloride solution. Extraction was achieved in the usual manner to yield a residue of azo 2+3.

Step II. One column (20 cm × 80 cm; 8.4 kg of silicone-treated Celite) was used for further fractionation to yield azo 2+3A and azo 2+3B.

Step IIIA. Fractionation of azo 2+3A on anhydrous Na_2SO_4 yielded three azo pigment fractions (azo $2+3A_1$, azo $2+3A_2$ and azo $2+3A_3$). Azo $2+3A_3$ was very faintly coloured, and was discarded. Azo $2+3A_1$ and azo $2+3A_2$ were incompletely separated. The front zone, consisting mainly of azo $2+3A_1$, was eluted and rechromatographed on anhydrous Na_2SO_4 with the use of chloroform. Azo $2+3A_1$ was now clearly separated from a faint zone of azo $2+3A_2$. The former was collected, evaporated to dryness and crystallized twice from hot methanol.

Azo $2+3A_2$ fractions from both columns were combined and rechromatographed on anhydrous Na_2SO_4. A small amount of residual azo $2+3A_1$ was removed by elution with chloroform and discarded. Azo $2+3A_2$ was then eluted with chloroform–ethyl acetate (5:3, v/v), and crystallized from hot methanol–water (3:1, v/v). Surprisingly, the last column also contained some azo $2+3A_3$, although this fraction had been completely removed during the first chromatographic run. Elution with methanol–acetic acid yielded only traces of azo $2+3A_3$. The yields of the compounds azo $2+3A_1$ and azo $2+3A_2$ are given in Table 1.

Step IIIB. Azo 2+3B was chromatographed on a column (9.8 cm × 190 cm) prepared from 4.4 kg of silicone-treated Celite. Azo $2+3B_1$ could not be detected in either of the two bile pools used in these experiments, but otherwise the chromatographic patterns were as shown in Scheme 1. The fractions were processed further in the usual manner to yield the compounds azo $2+3B_2$ to azo $2+3B_6$. The yields are given in Table 1.

Isolation of the phenylazo derivatives of bile bilirubin fraction 1

Attempts were made to isolate the phenylazo derivatives of all bilirubin fractions simultaneously by the large-scale isolation procedure described above. However, with bilirubin fraction 1 a heavy brown oil formed on azo coupling and evaporation. No phenylazo derivative could be extracted from this oily residue. Conversely, minute amounts of the phenylazo derivatives of bilirubin fraction 1 were obtained with the small-scale isolation described below.

Step I. A 17 ml portion of filtered bile was chromatographed by the usual procedure on a column (12 cm × 30 cm) of silicone-treated Celite (1.0 kg). Bilirubin fraction 1 was scraped from the column, with care being taken not to contaminate this fraction with pigment fraction Y. Bilirubin fraction 1 was eluted with methanol and filtered by suction. To the filtrate were added 8.5 ml of a chilled solution of phenyldiazonium chloride freshly prepared from 40 mg of $NaNO_2$ in 10 ml of water, and 0.05 ml of aniline in 20 ml of 1.5 M-HCl. The reaction mixture was immediately evaporated to dryness at 35°C. The residue was dissolved in a mixture of water and ethyl acetate, and the aqueous layer was extracted with ethyl acetate. The combined extracts were filtered and evaporated to dryness at 25°C to yield a residue of azo 1.

Step II. Azo 1 was dissolved in 1.0 ml of the organic layer of the solvent system, pH 6.0, and the solution was vigorously shaken with 10 ml of 5 mM-phosphate buffer, pH 6.0. The resulting oily emulsion was applied to a column (5.3 cm × 14 cm) of silicone-treated Celite (100 g), and was chromatographed in the usual manner. Approximately equal amounts of azo 1A and azo 1B were thus separated from a heavy brown oil, which remained on top of the column. On addition of solid NaCl (1:10, w/v) to the eluate of azo 1B, a pigmented butanol layer separated out, and was evaporated to dryness. The residue was dissolved in a mixture of water and ethyl acetate, and the aqueous layer was exhaustively extracted with ethyl acetate. The combined extracts were washed with water and evaporated to dryness to yield a residue of azo 1B. Azo 1A was discarded, as the yield was too low for further fractionation.

Step IIIB. Azo 1B (from step II) was chromatographed in the usual manner on a column (1.8 cm × 15 cm) of silicone-treated Celite (15 g). Apart from a very faint band of azo $1A_B$, a single slow-moving azo pigment fraction was detectable. The latter, again termed azo 1B, was eluted and filtered in the usual manner. The filtrate was evaporated to dryness as described for azo $2B_2$ to yield minute amounts of the compound azo 1B.

Chromatographic analysis of azo 1*B*

Azo 1B (from isolation step IIIB) was analysed by co-chromatography with the compounds azo $3B_2$ and azo $3B_3$ respectively. The chromatographic system described for step IIIB of the isolation procedure was used.

Azo 1B was dissolved in a small volume of mobile phase, pH 3.4, and the resulting solution was divided into two parts. A few crystals of azo $3B_2$ were dissolved in one half and azo $3B_3$ was dissolved in the other. Each of the two solutions was chromatographed on a column (1.8 cm × 10 cm) prepared from 10 g of silicone-treated Celite. A single azo pigment band appeared on the column that carried the mixture of azo 1B and azo $3B_2$, whereas two bands were visible with the mixture of azo 1B and azo $3B_3$.

DISCUSSION

Previous investigations (Billing *et al.* 1957; Schmid, 1957) have shown that the azo pigments derived from bilirubin of serum and bile can be separated into a non-polar fraction (azo pigment A) and a polar fraction (azo pigment B). These were characterized as the azo derivatives of unconjugated and conjugated bilirubin respectively. As shown in an accompanying paper (Kuenzle, 1970*b*), this characterization is essentially correct, all B pigments being derivatives of conjugated bilirubin, and the bulk of the A pigments (azo $2A_1$ and azo $3A_1$ being derivatives of unconjugated bilirubin. However, approx. 10% of the A pigments are derivatives of conjugated bilirubin (azo $2A_2$ and azo $3A_2$).

Various authors (e.g. Tenhunen, 1965) have claimed to have separated more than two azo pigments from human bile by t.l.c. and paper

chromatography. However, such pigments have never been isolated in a sufficiently pure state to permit their conclusive identification, and analytical data given must therefore be treated with reservation. In contrast, with the method presented in this paper, the isolation of several reasonably pure phenylazo derivatives of bilirubin has been achieved, and the structures of the main pigments have been elucidated (Kuenzle, 1970*c*).

At first sight it would seem that a total of 23 azo pigment fractions had been isolated by the above procedure. However, further examination decreases this number to eight (pigments azo A_1, azo A_2 and azo B_1 to azo B_6), mainly because the pigments derived from the three bilirubin fractions prove to be identical. A further decrease in the number of individual compounds is due to the finding that some of them arise from other pigments either by hydrolysis (all azo A_B pigments) or by degradation (all azo A_3 pigments). This is discussed below.

It is evident from Scheme 1 that the results of the fractionation steps IIIA and IIIB respectively are identical for azo pigments derived from bilirubin fractions 2 and 3. Results are not only identical with regard to the number and the chromatographic mobilities of the azo pigment fractions but also with regard to their relative concentrations. Identity of the chromatographic mobilities is evident from the results obtained with the phenylazo derivatives of the combined bilirubin fractions 2 and 3. In these experiments the chromatographic patterns proved to be identical with that observed with the azo pigments derived from the individual bilirubin fractions, except for the absence of the pigment azo $2+3B_1$. Therefore it is reasonable to assume that correspondingly numbered azo pigments from bilirubin fractions 2 and 3 (e.g. azo $2A_1$ and azo $3A_1$, and azo $2B_1$ and azo $3B_1$) are structurally identical.

The numbering system used to designate the azo pigments derived from bilirubin fractions 2 and 3 also seems to be applicable to the phenylazo derivatives of bilirubin fraction 1. The result of the chromatographic analysis of azo 1B (co-chromatography with azo $3B_2$) suggests that this pigment is of the B_2 type. It must be noted, however, that the bile sample used to isolate azo 1B did not contain the pigment azo $3B_1$, and it may well be that bilirubin fraction 1 would also have yielded an azo pigment of the B_1 type (along with the B_2 pigment) if such a pigment had been present in the coupled bilirubin fraction 3 of the bile sample investigated. The characterization of the fraction azo 1A has been prevented by the very low yield. However, there is little doubt of its identity with the fractions azo 2A and azo 3A.

The finding that bilirubin fractions 2 and 3 yield identical derivatives does not imply the identity of the two parent fractions. The difference rests in their respective yields of azo A and azo B pigments in step II. Approximately equal amounts of azo 2A and azo 2B are obtained from bilirubin fraction 2, whereas a ratio of approx. 0.1% of azo 3A to 99.9% of azo 3B is observed with bilirubin fraction 3. This finding is in accord with the results of earlier experiments (Kuenzle *et al.* 1966*b*). These have led to the characterization of bilirubin fraction 2 as being either a monoconjugated bilirubin or an equimolar complex of unconjugated and diconjugated bilirubin. The best available information (Billing *et al.* 1957; Nosslin, 1960; Gregory & Watson, 1962*a*; Gregory, 1963; Schalm, 1962; Weber, Schalm & Witmans, 1963) is in favour of the latter interpretation. However, in the light of the present investigation, it may be necessary to re-define such a complex as consisting of equal amounts of non-polar and polar bilirubin compounds. On azo coupling, non-polar bilirubin compounds would yield the pigments azo $2A_1$ and azo $2A_2$, whereas polar bilirubin compounds would give rise to the six azo B pigments (azo $2B_1$ to azo $2B_6$). A similar interpretation is suggested for bilirubin fraction 1. However, some modification would be necessary, since coupling yields only one azo B pigment together with an unspecified number of azo A pigments.

Bilirubin fraction 3 has been characterized as diconjugated bilirubin (Kuenzle *et al.* 1966*b*). The small amount of azo 3A observed in step II does not necessarily invalidate this interpretation, since the A pigments might arise from hydrolysis of the B pigments during the preceding steps of the isolation procedure. It is shown in an accompanying paper (Kuenzle, 1970*c*) that A pigments in fact are obtained on alkaline hydrolysis of the B pigments. Spontaneous hydrolysis had already been observed occasionally in previous experiments (Kuenzle *et al.* 1966*b*). Therefore it seems reasonable to assume that some hydrolysis also occurs with the present isolation procedure, thus accounting for the occurrence of the A pigments in the diconjugated bilirubin fraction 3.

The repeated occurrence of A pigments in step IIIB (azo $1A_B$, azo $2A_B$ and azo $3A_B$) and in subsequent rechromatographies of the individual B pigments is also attributed to some hydrolysis of B pigments. Examination of azo $2A_B$ and azo $3A_B$ on columns of anhydrous sodium sulphate showed both to be mainly composed of an azo pigment possessing the same chromatographic property as azo $2A_1$ and azo $3A_1$; but yields were too low to detect any of the other A pigments normally associated with azo $2A_1$ and azo $3A_1$.

The low yields of the pigments azo $2A_3$ and azo $3A_3$ prevented their conclusive identification. However, it is thought that these pigments are not

derivatives of bilirubin compounds actually present in bile but rather represent degradation products of azo $2A_2$ and azo $3A_2$ respectively. This interpretation stems from the observation that rechromatography of azo $2+3A_2$ led to the reappearance of azo $2+3A_3$, although this fraction had been completely removed during a first chromatographic run. This finding is hardly compatible with the presence of an individual parent bilirubin compound in bile.

The yields of the azo pigments isolated by the present method compare favourably with the azo pigment B yield obtained by Billing *et al.* (1957), which was less than 5%. The present yield of the total azo 3B pigments from bile pool I was calculated to be 4.3% of the theoretical value. The computation was based on the original concentration of bilirubin fraction 3 in bile pool I (see under 'Quantitative analysis of bilirubin fractions' in the Experimental and Results section), and on the molecular weights of bilirubin (mol.wt. 584.7) and of the pigments azo $3B_4$ (mol.wt. 728.7), azo $3B_5$ (mol. wt. 742.7) and azo $3B_6$ (mol.wt. 728.7) (Kuenzle, 1970*c*). Similarly the yield of the azo 2B pigments was calculated to be approx. 1% of the theoretical. The low yield of the azo 2B pigments is mainly due to losses that occurred on rechromatography of the isolated pigments. The yield of the azo 2A pigments was approx. 4% of the theoretical, with the computation being based on the molecular weight of azo $2A_1$ (mol.wt. 390.4; Kuenzle, 1970*c*).

Obviously the present method cannot be used for the determination of absolute quantities of the various azo pigments obtained from bile. Because of the losses occurring during manipulation the determination of relative proportions would also be liable to serious errors if pigments isolated by different techniques were to be compared (e.g. azo 2A versus azo 2B). However, the relative abundance of similar pigments present on a single chromatography column might be determined with reasonable accuracy, provided that the technique is suitably adjusted to such demands. Although no such modification has been devised in the present investigation, a tentative assignment of relative amounts of azo A and azo B pigments has been ventured on the basis of their respective yields. The values thus computed are given in Table 1. However, it must be stated that the composition of different bile specimens is expected to vary to some extent as is evident from the presence of the azo B_1 pigment in only one of the bile pools analysed in this investigation.

I express my gratitude to Professor C. H. Eugster for letting me pursue my own research project in his laboratory. I also thank Professor J. R. Rüttner for his interest in the present investigation. The repeated supply of large amounts of human hepatic bile by (the late) Dr E. Kaiser and by Dr M. Landolt is gratefully acknowledged. I am also indebted to Dr C. L. Green for reviewing the manuscript. This study was supported by a research grant from Fritz Hoffmann–La Roche Stiftung, Basle, Switzerland.

REFERENCES

Billing, B. H., Cole, P. G. & Lathe, G. H. (1957). *Biochem. J.* **65**, 774.

Fischer, H. & Plieninger, H. (1942). *Hoppe-Seyler's Z. physiol. Chem.* **274**, 231

Fog, J. (1964). *Scand. J. clin. Lab. Invest.* **16**, 49.

Gregory, C. H. (1963). *J. Lab. clin. Med.* **61**, 917.

Gregory, C. H. & Watson, C. J. (1962*a*). *J. Lab. clin. Med.* **60**, 1.

Gregory, C. H. & Watson, C. J. (1962*b*). *J. Lab. clin. Med.* **60**, 17.

Isselbacher, K. J. & McCarthy, E. A. (1959). *J. clin. Invest.* **38**, 645.

Jirsa, M., Ledvina, M. & Večerek, B. (1958). *Hoppe-Seyler's Z. physiol. Chem.* **311**, 93.

Jirsa, M. & Večerek, B. (1958). *Hoppe-Seyler's Z. physiol. Chem.* **311**, 87.

Kuenzle, C. C. (1970*a*). *Chimia*, **24**, 199.

Kuenzle, C. C. (1970*b*). *Biochem. J.* **119**, 395.

Kuenzle, C. C. (1970*c*). *Biochem. J.* **119**, 411.

Kuenzle, C. C., Maier, C. & Rüttner, J. R. (1966*b*). *J. Lab. clin. Med.* **67**, 294.

Kuenzle, C. C., Sommerhalder, M., Rüttner, J. R. & Maier, C. (1966*a*). *J. Lab. clin. Med.* **67**, 282.

Noir, B. A., Groszman, R. J. & DeWalz, A. T. (1966). *Biochim. biophys. Acta*, **117**, 297.

Nosslin, B. (1960). *Scand. J. clin. Lab. Invest.* **12** (Suppl.), 49.

Schachter, D. (1957). *Science, N.Y.*, **126**, 507.

Schalm, L. (1962). *Revue int. Hépat.* **12**, 559.

Schmid, R. (1957). *J. biol. Chem.* **229**, 881.

Schoenfield, L. J., Bollman, J. L. & Hoffman, H. N. (1962). *J. clin. Invest.* **41**, 133.

Talafant, E. (1956). *Nature, Lond.*, **178**, 312.

Tenhunen, R. (1965). *Annls Med. exp. Biol. Fenn.* **43**, Suppl. 6.

van den Bergh, A. A. A. & Muller, P. (1916). *Biochem. Z.* **77**, 90.

Vegas, F. R. (1963). *Analyt. Biochem.* **5**, 465.

Weber, A. P. & Schalm, L. (1965). *Acta med. scand.* **177**, 519.

Weber, A. P., Schalm, L. & Witmans, J. (1963). *Acta med. scand.* **173**, 19.