Biochem. J. (1970) **119**, 411–435
Printed in Great Britain

Bilirubin Conjugates of Human Bile

THE EXCRETION OF BILIRUBIN AS THE ACYL GLYCOSIDES OF ALDOBIOURONIC ACID, PSEUDOALDOBIOURONIC ACID AND HEXURONOSYLHEXURONIC ACID, WITH A BRANCHED-CHAIN HEXURONIC ACID AS ONE OF THE COMPONENTS OF THE HEXURONOSYLHEXURONIDE

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(*Received* 19 *January* 1970)

Structure elucidations have been performed on the bilirubin conjugates isolated from human hepatic bile as the phenylazo derivatives. The major bilirubin conjugates are excreted, not as was formerly thought in the form of glucuronides, but as the acyl glycosides of aldobiouronic acid, pseudoaldobiouronic acid and hexuronosylhexuronic acid. The isolated aldobiouronides are proposed to have the structures of an acyl 6-*O*-hexopyranosyluronic acid-hexopyranoside, an acyl 4-*O*-hexofuranosyluronic acid-D-glucopyranoside, and an acyl 4-*O*-β-D-glucofuranosyluronic acid-D-glucopyranoside respectively, with the acyl radicals being those of the phenylazo derivative of bilirubin. The pseudoaldobiouronide is suggested to be the acyl 4-*O*-α-D-glucofuranosyl-β-D-glucopyranosiduronic acid, with the acyl radical being that of the phenylazo derivative of vinylneoxanthobilirubinic acid. The hexuronosylhexuronide presumably is the acyl 4-*O*-(3-*C*-hydroxymethylribofuranosyluronic acid)-β-D-glucopyranosiduronic acid, with the acyl radical being that of the phenylazo derivative of bilirubin. The 3-*C*-hydroxymethylriburonic acid, isolated as one of the components of the hexuronosylhexuronide, is the first natural branched-chain hexuronic acid to be detected, and the first branched-chain sugar ever detected in humans.

Various bilirubin compounds have been shown to occur in human hepatic bile, and their isolation as the phenylazo derivatives has been reported (Kuenzle, 1970*b*). The present paper is an account of the structure elucidations performed on the isolated phenylazo pigments. Structural analysis was facilitated by spectroscopic evidence derived from model bilirubin compounds (Kuenzle, 1970*c*).

With the present work I propose to show that the major bilirubin conjugates of human bile occur, not as glucuronides (Talafant, 1956; Billing, Cole & Lathe, 1957; Schmid, 1957), but as the acyl glycosides of aldobiouronic acid, pseudoaldobiouronic acid and hexuronosylhexuronic acid. The conjugation of human metabolites with acidic disaccharides of the above type has not been reported previously. Moreover, a novel branched-chain hexuronic acid was detected as one of the components of the isolated hexuronosylhexuronide. Branched-chain sugars are known to occur in plants and micro-organisms (Shafizadeh, 1956; Berry, 1963; Grisebach, 1967; Okuda, Suzuki & Suzuki, 1967, 1968; Gustine & Kindel, 1969), but have not previously been found in animals nor humans.

In this paper the following trivial names have been employed: cellobiouronic acid, 4-*O*-β-D-glucopyranosyluronic acid-D-glucose; gentiobiouronic acid, 6-*O*-β-D-glucopyranosyluronic acid-D-glucose; kojibiose, 2-*O*-α-D-glucopyranosyl-D-glucose; laminaribiose, 3-*O*-β-D-glucopyranosyl-D-glucose; pseudomaltobiouronic acid, 4-*O*-α-D-glucopyranosyl-D-glucuronic acid.

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

EXPERIMENTAL

Materials

Azo pigments A_1, A_2 and B_1 to B_6. Structure elucidations were performed on the phenylazo derivatives of the bilirubin compounds isolated from the bile pools I, II and III as described in an accompanying paper (Kuenzle, 1970*b*). Throughout this work the isolated phenylazo derivatives are designated according to their chromatographic mobilities as azo pigments A_1 and A_2 and azo

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pigments B_1 to B_6. These designations are used regardless of whether the pigments were obtained from the individual or the combined bilirubin fractions 2 and 3 (Kuenzle, 1970*b*). Thus, for example, the term azo pigment A_1 stands for either of the pigments formerly (Kuenzle, 1970*b*) denoted as pigments azo $2A_1$, azo $3A_1$ and azo $2+3A_1$. The pigments from bilirubin fraction 1 were not analysed in this work.

Phenylazo derivative of 3,18-*di*(*ethylidene sulphate*)-2,7,13,17-*tetramethylbiladiene*-ac-8,12-*dipropionic acid*. This compound was prepared as reported in an accompanying paper (Kuenzle, 1970*c*) by treatment of bilirubin with acetic anhydride $-H_2SO_4$ followed by coupling with phenyldiazonium chloride. The pigment is referred to below as the phenylazo derivative of synthetic bilirubin sulphate.

Sugars. D-Glucuronic acid, D-glucuronolactone and D-glucose were purchased from Fluka A.G., Buchs SG, Switzerland. L-Iduronolactone was prepared from dermatan sulphate (which was a gift from Dr K. von Berlepsch, F. Hoffmann–La Roche Ltd., Basle, Switzerland) by hydrolysis (1 M-H_2SO_4 at 100°C for 2 h in a sealed glass tube) followed by passage through Dowex 1 (X10; CO_3^{2-} form) and Dowex 50W (X12; H^+ form). The following sugars were also gifts: D-mannuronolactone (Professor H. Neukom, Eidg. Technische Hochschule, Zürich, Switzerland), L-guluronolactone and L-guluronic acid as an equilibrium mixture in aqueous solution (Dr A. Haug, Institute of Seaweed Research, Trondheim, Norway), cellobiouronic acid (Professor G. Jayme, Technische Hochschule, Darmstadt, Germany), β-hepta-*O*-acetylgentiobiouronic acid methyl ester (Professor B. Helferich, University of Bonn, Bonn, Germany), kojibiose (Dr K. Matsuda, Tohoku University, Sendai, Japan), laminaribiose (Dr W. J. Whelan, University of Miami, Miami, Fla., U.S.A.) and hepta-*O*-acetylpseudomaltobiouronic acid (Dr Y. Hirasaka, Chugai Pharmaceutical Co., Tokyo, Japan).

Enzymes. β-Glucuronidase (β-D-glucuronide glucuronohydrolase, EC 3.2.1.31, from bovine liver, type B-3) was purchased from Sigma Chemical Co., St Louis, Mo., U.S.A. β-Glucosidase (β-D-glucoside glucohydrolase, EC 3.2.1.21, from almonds) was purchased from Fluka A.G. α-Glucosidase [sucrose glucohydrolase, EC not listed, from rabbit small intestine (Kolínská & Semenza, 1967; Semenza, Curtius, Kolínská & Müller, 1967; Cummins, Gitzelmann, Lindenmann & Semenza, 1968)] was a gift from Professor R. Gitzelmann, Department of Pediatrics, University of Zürich, Zürich, Switzerland.

Reagents. All chemicals used were reagent grade, and were purchased either from E. Merck A. G., Darmstadt, Germany, or from Fluka A. G.

Apparatus and chromatography systems

N.m.r. spectra. Spectra were recorded at 100 MHz (0.05 M solutions) with a Varian HR-100 spectrometer. Solvents were [$^2H_6$]dimethyl sulphoxide with the azo pigments, and deuterium oxide with the compounds R_5 and R_6. Tetramethylsilane was used as an internal and external standard respectively. Chemical shifts are given in p.p.m. (±0.03) relative to tetramethylsilane = 0.

I.r. spectra. Spectra were recorded from solids in Nujol mulls unless otherwise stated. The recording instruments were a Perkin–Elmer model 21 spectrograph equipped with NaCl optics and a Beckman model IR8 infrared spectrometer.

Optical spectra. Spectra of all azo pigments were recorded as described in an accompanying paper (Kuenzle, 1970*c*).

Optical rotation. Measurements were taken with a Carl Zeiss photoelectric precision polarimeter (±0.005°) in microcells (0.3 ml) of 2 cm light-path.

Mass spectra. Spectra were recorded with an LKB gas chromatograph–mass spectrometer type 9000 (70 eV) under the following conditions.

System MS–1 for trimethylsilylated sugars was as follows: glass column, 2.5 m length, 4 mm inside diameter; liquid phase, 1% SE–30 on Chromosorb W; T_c (column temperature), 155°C; T_j (injector-block temperature), 220°C; T_s (separator temperature), 250°C; $T_{i.s.}$ (ion-source temperature), 270°C; carrier gas, He, 30 ml/min; flame ionization detector. Samples obtained from aqueous solutions were trimethylsilylated with pyridine–hexamethyldisilazane–chlorotrimethylsilane (10:2:1, by vol.) (Sweeley, Bentley, Makita & Wells, 1963), and the reaction mixtures were directly injected on to the column. The crystalline sugars used as reference compounds were left to mutarotate in water for 1 h at 40°C, and were dried in a high vacuum over P_2O_5 before trimethylsilylation.

System MS–2 for methylated aldobiouronic acid methyl esters was as follows: glass column, 2.5 m length, 4 mm inside diameter; liquid phase, 1% SE–30 on Chromosorb W; T_c, 206°C; T_j, 245°C; T_s, 250°C; $T_{i.s.}$, 250°C; carrier gas, He, 32 ml/min; flame ionization detector. Samples were dissolved in dichloromethane and were injected on to the column.

G.l.c. A Perkin–Elmer F 20 Fraktometer equipped with a Hitachi recorder was used with packed columns, and a Carlo Erba Fraktovap was used with capillary columns. Capillary columns were prepared by the method of Grob (1968). Samples were prepared for injection as described above (see under 'Mass spectra').

System GLC–1 for trimethylsilylated sugars was as follows: packed glass column, 2 m length, 2.7 mm inside diameter; liquid phase, 3% SE–30 on Gas-Chrom P; T_c, 155°C; T_j, 240°C; carrier gas, N_2, 22 ml/min; flame ionization detector.

System GLC–2 for trimethylsilylated sugars was as follows: glass capillary, 30 m length, 0.3 mm inside diameter; liquid phase, SE–52; T_c, 140°C; T_j, 265°C; carrier gas, H_2 at a pressure of 1.00 kg/cm^2; flame ionization detector.

Preparative t.l.c. of methylated and acetylated carbohydrates. T.l.c. was performed on glass plates coated with silica gel PF_{254} (E. Merck A. G.). The eluents employed were benzene–ethanol (5:1, v/v) with the methylated carbohydrates (Wallenfels, Bechtler, Kuhn, Trischmann & Egge, 1963) and chloroform–ethyl acetate (5:3, v/v) with the acetylated carbohydrates. To detect the methylated compounds, test strips were sprayed with H_2SO_4 and were charred for 15 min at 110°C. No spraying was needed to detect the acetylated compounds, since they stained faintly yellow on drying of the plates in a stream of warm air.

Paper chromatography. The following chromatography systems and spraying reagents were used.

System PC–1 was as follows: upper phase of butan-1-ol–

formic acid–water (100:23:77, by vol.), descending on Whatman no. 1 paper (Edington & Percival, 1955).

System PC–2 was as follows: *n*-butyl acetate–acetic acid–butan-1-ol–methanol–water (3:2:2:1:1, by vol.), descending on acid-washed Schleicher und Schuell no. 2043a paper (Masamune & Satake, 1959).

System PC–3 was as follows: 0.2M-acetic acid, ascending on sheets of DEAE-cellulose (Whatman DE20 anion-exchange paper).

Chromatograms were sprayed with aniline phthalate for detection of reducing sugars. Amino acids were sought by spraying with ninhydrin.

Reverse-phase partition chromatography of the azo pigments. This was carried out on columns (0.75 cm × 30 cm) of silicone-treated Celite with the use of the solvent system octan-1-ol–di-isopropyl ether–ethyl acetate–methanol–0.2M-acetic acid (1:2:2:3:4, by vol) (Kuenzle, 1970*b*).

Methods

Alkali-stability of azo pigments B_1 to B_6. A few crystals of the azo pigments were dissolved in 50μl of dimethyl sulphoxide. Then 3ml of 0.05M-NaOH was added, and the solutions were left to stand for 0.5h at 20°C. The solutions were adjusted to pH5 with 0.05M-HCl, and were extracted with 4ml of chloroform. With the azo pigments B_2 to B_6, all of the red pigments were present in the chloroform layer after a single extraction. The extracts were evaporated to dryness and were further dried in a high vacuum. (Throughout this work evaporation of solvents was performed in a rotary evaporator with the temperature being kept as low as possible.) The residues were dissolved in the minimum volume of chloroform, and were chromatographed on columns (1cm × 6cm) of anhydrous Na_2SO_4 with the use of chloroform as an eluent (Kuenzle, 1970*b*,*c*). When the azo pigment B_1 was subjected to the above treatment, the chloroform layer remained colourless. Therefore the red pigments were extracted with butan-1-ol. The extract was washed with 0.2M-acetic acid and evaporated to dryness by azeotropic distillation with water (Kuenzle, 1970*b*). The residue was dried in a high vacuum and subjected to the reverse-phase partition chromatography described above under 'Apparatus and chromatography systems'.

Assays for phosphorus and sulphur. Spot tests for phosphorus were performed on a semiquantitative basis by fusion with CaO followed by reaction with ammonium molybdate–benzidine (Feigl, 1960). Quantitative sulphur determinations by the methods of Wagner (1957) were performed with compounds R_5 and R_6. With all other compounds qualitative tests for sulphur were carried out by fusion with sodium followed by acidification and reaction with metallic silver.

Assay for hexuronic acids. Uronic acids were determined by an adaption of the naphtharesorcinol method for total glucuronic acid (Fishman & Green, 1955). The azo pigments were dissolved in 0.05M-NaOH. Portions were transferred to the reaction tubes, and were immediately neutralized with 0.20M-HCl. Assays were run in triplicate. Standard solutions were prepared from D-glucuronic acid, which was dissolved in 0.05M-NaOH, and was neutralized as described above.

Alkaline hydrolysis of azo pigments B_4 to B_6 and isolation of the cleaved moieties. The azo pigments were dissolved in aqueous 0.1M-NH_3 (1mg of azo pigment/ml) and the solutions were shaken for 1h at 20°C. They were evaporated to dryness by azeotropic distillation with butan-1-ol (Kuenzle, 1970*b*). The residues were further dried in a high vacuum and redissolved by shaking with ethyl acetate–0.1M-acetic acid (1:1, v/v). The layers were allowed to separate and were processed individually.

To isolate the pigment moieties of the azo pigments, the organic layers were washed twice with water and evaporated to dryness. After drying in a high vacuum, the residues were dissolved in chloroform and passed through columns of anhydrous Na_2SO_4 (Kuenzle, 1970*b*,*c*). The bulk of the red pigments moved with the solvent front, and a minor fraction remained just below the surface of the column. The former were collected and were evaporated to dryness. Crystallization was effected from hot methanol to yield the *pigment moieties of azo pigments B_4, B_5 and B_6* respectively (approx. 30mg from 100mg of starting material).

To isolate the non-pigmented moieties of the azo pigments, the aqueous layers and the washings of the above extractions were washed exhaustively with butan-1-ol to remove any residual pigments. After evaporation, the residues were redissolved in the minimum volume of water. The solutions were filtered and evaporated to dryness. After further drying in a high vacuum, *compounds R_4, R_5 and R_6* (from the azo pigments B_4, B_5 and B_6 respectively) were obtained as brownish glasses. Yields were approx. 30mg of substance/100mg of starting material. Attempts to crystallize the compounds from ethanol, methanol or acetone were unsuccessful.

Incubation of the azo pigments with β-glucuronidase. Azo pigment B_1 (0.3mg) and azo pigments B_4, B_5 and B_6 (1.0mg) were dissolved in 25μl of dimethyl sulphoxide, and 2.5ml of 0.05M-sodium acetate buffer, pH5.0, was added. The solutions were filtered and 2.0ml portions were transferred to the reaction tubes. β-Glucuronidase [1200 Fishman units; 1 Fishman unit liberates 1μg of phenolphthalein/h from phenolphthalein glucuronide at pH5 and 37°C (Fishman, Springer & Brunetti, 1948)] in the above buffer (0.5ml) was added, and the reaction mixtures were incubated for 3.5h at 37°C under N_2. The enzyme was precipitated with dry ethanol (7.5ml) and removed by filtration. The filtrates were evaporated to dryness and dried further in a high vacuum. The pigments were dissolved in chloroform and chromatographed on anhydrous Na_2SO_4 (Kuenzle, 1970*b*,*c*). In all cases, control experiments were carried out with heat-inactivated enzyme (100°C for 10min).

With azo pigment B_4 two additional experiments were carried out. In the first, incubation with β-glucuronidase was performed as above, but after 3.5h the reaction mixture was exhaustively extracted with butan-1-ol. The extract was evaporated to dryness by azeotropic distillation with water (Kuenzle, 1970*b*) and dried in a high vacuum. The pigmented residue was then subjected to the reverse-phase partition chromatography described above under 'Apparatus and chromatography systems'. In the second experiment, incubation with β-glucuronidase was begun as above, but four additional enzyme portions (1200 Fishman units each) were added at hourly intervals. Enzymic hydrolysis was then allowed to proceed overnight. The reaction mixture was extracted with butan-1-ol, and was subjected to reverse-phase partition chromatography as described above.

Incubation of compound R_4 with β-glucuronidase. Compound R_4 (1mg) was dissolved in 2ml of 0.05M-sodium acetate buffer, pH5.0. β-Glucuronidase (1500 Fishman units) was added, and the reaction mixture was incubated for 24h at 37°C under N_2. The enzyme was removed by heating at 100°C for 10min followed by filtration, and the filtrate was evaporated to dryness. The residue was taken up in aqueous 0.05M-NH_3 and was again evaporated to dryness. It was further dried in a high vacuum over P_2O_5 before trimethylsilylation and g.l.c. (see above under 'Apparatus and chromatography systems').

Incubation of azo pigments B_4 and B_6 with glucosidases. Azo pigment B_4 (1mg) was treated with β-glucuronidase (1200 Fishman units at 37°C for 24h; see above), and was extracted with ethyl acetate before incubation with either α- or β-glucosidase. The pigment obtained on evaporation of the ethyl acetate extract was redissolved in 120μl of dimethyl sulphoxide, and 50μl portions were transferred to two reaction tubes. These contained α-glucosidase [0.02 unit; 1 unit of enzyme cleaves 1μmol of sucrose/min at pH6.8 and 37°C (Kolínská & Semenza, 1967)] in 2ml of 0.02M-sodium phosphate buffer, pH6.4, and β-glucosidase (1mg) in 2ml of 0.02M-sodium phosphate buffer, pH5.6, respectively. The reaction mixtures were held for 20h at 37°C under N_2. Dry ethanol (8ml) was added, and the solutions were filtered and evaporated to dryness. After further drying in a high vacuum, the pigments were taken up in chloroform and passed through columns of anhydrous Na_2SO_4 (Kuenzle, 1970*b*,*c*).

Azo pigment B_6 (0.3mg) was dissolved in 25μl of dimethyl sulphoxide, and 10μl portions were transferred to two reaction tubes. These contained α-glucosidase (0.04 unit) and β-glucosidase (0.1mg) respectively in 100μl of the above buffers. Incubations were carried out for 1h at 37°C under N_2. The reaction mixtures were extracted with butan-1-ol, and were evaporated to dryness by azeotropic distillation with water (Kuenzle, 1970*b*). After further drying in a high vacuum, the pigments were subjected to the reverse-phase partition chromatography described above under 'Apparatus and chromatography systems'.

Incubation of compound R_6 with α-glucosidase. Compound R_6 (0.2mg) was dissolved in water and passed through a column (0.75cm × 3cm) of CM-cellulose (type CM ion-exchange cellulose; Serva Entwicklungslabor, Heidelberg, Germany) to remove NH_4^+ ions. The eluate was evaporated to dryness, and the residue was redissolved in 0.6ml of water that had been adjusted to pH6.8 by the addition of $NaHCO_3$. [NH_4^+ is a strong inhibitor of the enzyme, and Na^+ is a non-essential activator (Kolínská & Semenza, 1967).] α-Glucosidase (0.15 unit) was added, and the solution was incubated for 1h at 37°C under N_2. The enzyme was removed by heating at 100°C for 10min followed by filtration, and the filtrate was evaporated to dryness. The residue was further dried in a high vacuum before trimethylsilylation and g.l.c. (see above under 'Apparatus and chromatography systems').

Incubation of compound R_6 with β-glucosidase. Compound R_6 (0.2mg) was dissolved in 4ml of 0.05M-sodium acetate buffer, pH5.2, and β-glucosidase (0.5mg) was added. Incubation was carried out for 6h at 37°C under N_2. Further processing was effected as described for α-glucosidase (see above).

Acid hydrolysis of compounds R_4, R_5 and R_6. Acid hydrolysis was performed in sealed glass tubes under various hydrolytic conditions.

(*a*) Analysis for amino acids. Hydrolysis was carried out with 6M-HCl for 16h at 110°C. The hydrolysates were diluted with water and evaporated to dryness with the repeated addition of more water. The residues were further dried in a high vacuum over KOH before paper chromatography (systems PC–1 and PC–2; see above under 'Apparatus and chromatography systems').

(*b*) Analysis for sugars. Compound R_5 was converted into the free acid by passage through Dowex 50W (X12; H^+ form) before hydrolysis. Compounds R_4 and R_6 were hydrolysed as the ammonium salts. Hydrolysis was effected with either 1M-HCl (compounds R_4 and R_6) or 0.1M-HCl (compound R_5) for 1h at 100°C, and the hydrolysates were evaporated to dryness as described above. Generally the residues were redissolved in aqueous 0.05M-NH_3 to convert hexuronolactones into the corresponding hexuronate salts. However, this treatment proved to be deleterious with the hydrolysate of compound R_5 (see the Discussion section). The solutions were again evaporated to dryness and were further dried in a high vacuum before trimethylsilylation and g.l.c. (see above under 'Apparatus and chromatography systems'). In one instance the compound R_5 was hydrolysed with 0.01M-HCl for 0.5h at 100°C, and the hydrolysate was adjusted to pH6 by the dropwise addition of 0.01M-$NaHCO_3$. The solution was evaporated to dryness and was further dried in a high vacuum over P_2O_5 before trimethylsilylation and g.l.c.

Esterification and O-methylation of compound R_4. Compound R_4 (10mg) was dissolved in water and was converted into the free acid by passage through Dowex 50W (X12; H^+ form). The eluate was evaporated to dryness to yield 8.3mg of a brownish glass. The latter was redissolved in water (0.1ml), and cold methanol (3ml) was added followed by excess of diazomethane (ether solution). Esterification was allowed to proceed for 10min at 0°C, and the reaction mixture was filtered and evaporated to dryness. The glassy residue was further dried in a high vacuum over P_2O_5. *O*-Methylation was then performed at 20°C in dry dimethyl sulphoxide–*NN*-dimethylformamide (1:1, v/v) with dimethyl sulphate, BaO and $Ba(OH)_2$ (Kuhn & Trischmann, 1963). More dimethyl sulphate and BaO was added after 8h, and the reaction was allowed to proceed overnight. Excess of dimethyl sulphate was decomposed with aqueous NH_3 (sp.gr. 0.88), and the methylated products were extracted with chloroform (Kuhn & Trischmann, 1963). Preparative t.l.c. was then performed (see above under 'Apparatus and chromatography systems'). The major fraction (R_F 0.5) was scraped from the plate, and was eluted with chloroform. The fully methylated methyl ester derivative of compound R_4 was obtained in a yield of approx. 0.1mg.

Esterification and O-methylation of compounds R_5 and R_6. Several unsuccessful attempts were made to prepare the fully methylated methyl ester derivatives of compounds R_5 and R_6 respectively, either by the method described in the preceding paragraph or by esterification with diazomethane followed by methylation with methyl iodide and sodium hydride in *NN*-dimethylformamide (Diner, Sweet & Brown, 1966). T.l.c. invariably revealed the presence of large amounts of decomposition products that moved with the solvent front, but methylated sugar

derivatives were not detected. Methylation also failed to give the desired products when the fully acetylated methyl ester derivatives of compounds R_5 and R_6 were used as the starting materials (4.3mg and 3.7mg respectively; see the following paragraph).

Esterification and O-acetylation of compounds R_5 and R_6. Compounds R_5 (25mg) and R_6 (23mg) were converted into the free acids by passage through Dowex 50W (X12; H^+ form), and were esterified with diazomethane (see above under 'Esterification and *O*-methylation of compound R_4'). Acetylation was performed with 0.4ml of dry pyridine–acetic anhydride (1:1, v/v) for 3h at 20°C, and the reaction mixtures were evaporated to dryness in a high vacuum at 70°C (Weissmann & Meyer, 1954). The residue was purified by preparative t.l.c. (see above under 'Apparatus and chromatography systems'). The major fraction (R_F 0.6–0.7) was scraped from the plate and eluted with chloroform. The yellow glass obtained on evaporation was crystallized from dry ethanol (15 days at −20°C) to yield off-white crystals corresponding to the fully acetylated methyl ester derivatives of compounds R_5 (2.0mg) and R_6 (0.9mg) respectively. The mother liquors were evaporated to dryness to give additional amounts of the compounds (3.8mg and 2.8mg respectively).

Preparation of hepta-O-methylaldobiouronic acid methyl esters. Cellobiouronic acid and β-hepta-*O*-acetylgentiobiouronic acid methyl ester were used as the starting materials.

Cellobiouronic acid (32mg) was left to mutarotate in water (0.1ml) for 16h at 20°C, and after addition of 3ml of methanol was converted into the methyl ester with diazomethane. Methylation with dimethyl sulphate–BaO and purification by t.l.c. was performed as described for compound R_4. The yield of the hepta-*O*-methylcellobiouronic acid methyl ester was 4.8mg (11.4% on a molar basis).

β-Hepta-*O*-acetylgentiobiouronic acid methyl ester (35.6mg) was converted into gentiobiouronic acid by saponification with 12mM-sodium methoxide (16h at 0°C) (Helferich & Berger, 1957). The reaction mixture was evaporated to dryness, and the residue was left to mutarotate in water for 6h at 20°C before desalting with Dowex 50W (X12; H^+ form). Esterification followed by methylation and t.l.c. was performed as described for compound R_4 to yield 7.5mg (29.7% on a molar basis) of the hepta-*O*-methylgentiobiouronic acid methyl ester.

RESULTS

Azo pigments A_1 and A_2

Azo pigment A_1. This pigment was identified as the phenylazo derivative of bilirubin by elemental analysis and by spectroscopic methods [Found: C, 67.8; H, 6.2; N, 13.9 (corrected for 1.2% inorganic impurity present in the sample). Calc. for $C_{22}H_{22}N_4O_3$ (mol.wt. 390.43): C, 67.7; H, 5.7; N, 14.4%]. The optical spectrum showed absorption maxima at 270nm (ε 13500), 277nm (ε 13800), 327nm (ε 19500) and 514nm (ε 29300), in good agreement with the spectrum obtained from an authentic sample (Kuenzle, 1970c). The i.r. spectra of the isolated and the authentic sample were also virtually identical (Kuenzle, 1970c). Further, the isolated pigment showed all the n.m.r. signals characteristic of the phenylazo derivative of bilirubin (Kuenzle, 1970c), but differed from the latter in one aspect. Whereas the authentic sample consisted of equimolar amounts of the phenylazo derivatives of vinylneoxanthobilirubinic acid and isovinylneoxanthobilirubinic acid (Fig. 6), the isomer ratio was approx. 2:1 with the isolated compound (for a detailed discussion of the azo coupling reaction and of the isomer mixture thereby generated from bilirubin IXα see Kuenzle, 1970c).

Azo pigment A_2. Only small amounts of this compound were available, and its analysis was therefore restricted to spectroscopic methods. The optical spectrum showed absorption maxima at 327 and 514nm, and thus indicated the presence of the same chromophore as occurs in the phenylazo derivative of bilirubin (Kuenzle, 1970c). A plateau of relatively low intensity was detected in the range 270–280nm. Quantitative measurements were not taken, but the relative intensities of the three absorptions suggested an enhanced absorption in the u.v. region as compared with the phenylazo derivative of bilirubin.

The i.r. spectrum of azo pigment A_2 is shown in Fig. 1. The i.r. absorptions in the range 670–1680cm^{-1} were similar to the ones observed with the phenylazo derivative of bilirubin (Kuenzle, 1970c). In contrast, pronounced differences were detected in the range 1680–4000cm^{-1}. No absorption was found at 1712cm^{-1} (CO_2H with the phenylazo derivative of bilirubin). Instead, the absorption at 1738cm^{-1} suggested the presence of an ester function. A broad band at 3333cm^{-1} was indicative of OH and/or NH_2 groups.

The n.m.r. spectrum also resembled that of the phenylazo derivative of bilirubin (Kuenzle, 1970c), but the positions of some of the signals were slightly shifted (up to ±0.15p.p.m.). The signal at 11.84p.p.m. (4 protons with the phenylazo derivative of bilirubin) was very small, and could account for not more than 2 protons. The signals corresponding to both the vinyl- and the isovinylneoxanthobilirubinic acid isomer were detected in the spectrum, but the isomer ratio was approx. 2:1. A few signals appeared in the spectrum of azo pigment A_2 that could not be assigned to the protons of the pigment moiety. These signals were rather broad, and were positioned at 4.50, 5.06 and 5.26p.p.m. respectively. Most probably they belonged to an unidentified radical attached to the pigment moiety. No attempt was made to deduce the structure of this radical from the scarce information acquired, particularly in view of possibly undetected resonance lines buried beneath the solvent signals.

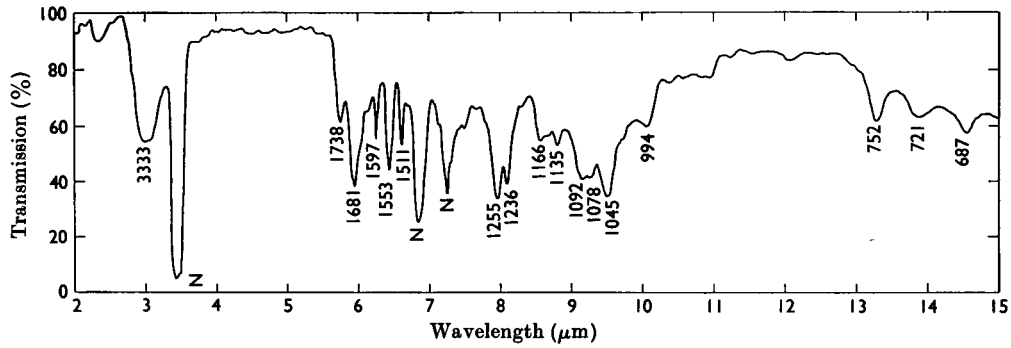

Fig. 1. I.r. spectrum of the azo pigment A_2 (Nujol mull). Positions of bands are given in cm^{-1}. Nujol absorptions are marked with N. Assignments are: O–H and/or N–H stretch at 3333 cm^{-1}, carbonyl stretch of ester group at 1738 cm^{-1} and carbonyl stretch of α-pyrrolone (amide I band) at 1681 cm^{-1}.

Azo pigments B_1 to B_6

Chromatography of azo pigment B_6 together with the phenylazo derivative of synthetic bilirubin sulphate. To rule out the possibility that one of the azo pigments isolated from human bile could be the phenylazo derivative of 3,18-di(ethylidene sulphate)-2,7,13,17-tetramethylbiladiene-*ac*-8,12-dipropionic acid, the most polar of the isolated azo pigments (azo pigment B_6) was chromatographed together with this synthetic sulphate compound. On reverse-phase partition chromatography columns the sulphate compound moved approximately three times as fast as the azo pigment B_6. It was thus shown not to be the derivative of a naturally occurring bilirubin conjugate.

Alkali-stability of azo pigments B_1 to B_6. Mild alkaline treatment (0.05M-sodium hydroxide for 0.5h at 20°C) of azo pigment B_1 did not change its solubility properties. The pigment remained insoluble in chloroform, and had the same mobility as the untreated pigment on reverse-phase partition chromatography columns. In contrast, the same treatment markedly altered the solubility characteristics of azo pigments B_2 to B_6. The pigments became extractable with chloroform, and on chromatography on anhydrous sodium sulphate proved to move at the same rate as the phenylazo derivative of bilirubin (Kuenzle, 1970c). Thus azo pigment B_1 was characterized as being alkali-stable, and azo pigments B_2 to B_6 were found to be alkali-labile.

Azo pigment B_1. Apart from being the only alkali-stable pigment of the B group (see the preceding paragraph), this compound also differed from all other pigments in its light-absorption properties. The optical spectrum showed absorption maxima at 270, 277, 312 and 503nm, and its shape strongly resembled the spectrum of the phenylazo derivative of mesobilirubin (Kuenzle 1970c). The i.r. spectrum (potassium bromide) was similar to the one shown in Fig. 2 for azo pigment B_6, but the bands were less well resolved, particularly in the carbonyl region. Therefore it was not possible to identify any specific carbonyl frequency. Azo pigment B_1 contained neither phosphorus nor sulphur, and the compound was not cleaved by β-glucuronidase.

Azo pigments B_2 and B_3. The alkali-lability of both compounds has already been mentioned above. The optical spectra showed a single absorption maximum at 514nm. Below 410nm the presence of u.v.-absorbing impurities caused a gradual increase of absorption. This prevented detection of any fine structure possibly present. The i.r. spectra (potassium bromide) were essentially as described for azo pigment B_1 with an equally low resolution of the bands. No other analyses were performed because of lack of material.

Azo pigment B_4. The optical spectrum of azo pigment B_4 showed the same absorption maxima (270, 277, 327 and 514nm) as the phenylazo derivative of bilirubin (Kuenzle, 1970c), and the shapes of both curves were virtually identical. This indicated that the chromophores were the same in both compounds. Therefore the colorimetric determination of the molecular weight of azo pigment B_4 was possible. The molecular weight was calculated from an extinction coefficient of 36.51 $g^{-1}cm^{-1}$ [corrected for 11.5% inorganic impurity (probably silica) present in the sample], as obtained from azo pigment B_4 at 514nm, and from the molar absorptivity (ϵ_{514} 29600) of the phenylazo derivative of bilirubin (Kuenzle, 1970c). The molecular weight thus determined was 810.

The i.r. spectrum (potassium bromide) of azo pigment B_4 revealed an ester carbonyl band at 1733 cm^{-1}, and was virtually indistinguishable

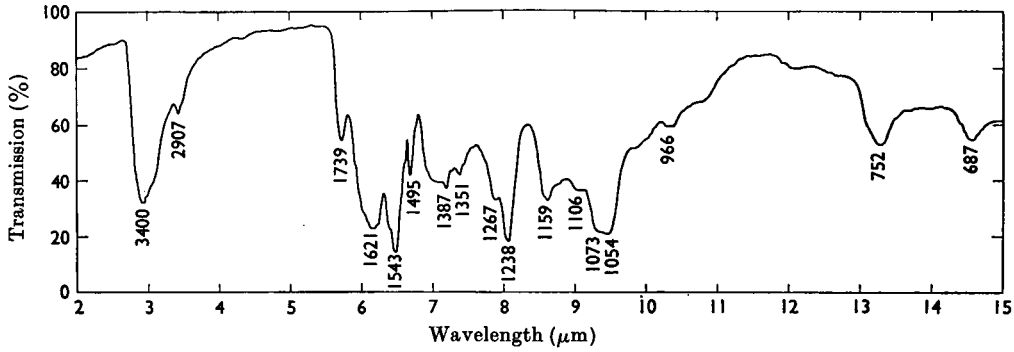

Fig. 2. I.r. spectrum (KBr) of the azo pigment B_6. Positions of bands are given in cm^{-1}. The broad band centred at 3400 cm^{-1} is a combination of several vibrations, i.e. O–H stretching modes of the carbohydrate hydroxyl groups, N–H stretch of the pyrrole and α-pyrrolone rings and N–H stretch of the protonated azo group ($-N{=}\overset{+}{N}H-$). CH_3, CH_2 and CH stretching frequencies are at 2907 cm^{-1}. Carbonyl stretch of ester group is at 1739 cm^{-1}. This ester band is specifically assigned to the acyl glycoside linkage since it is missing from the i.r. spectrum of compound R_6 (the substituent cleaved from the pigment moiety by mild alkaline hydrolysis). The broad absorption at 1621 cm^{-1} is a combination of several bands, i.e. carbonyl stretch of α-pyrrolone, carbonyl stretch of CO_2^- (hexuronate) and C=C stretch of the phenyl, pyrrole and α-pyrrolone rings. The complex absorption in the region 1200–1030 cm^{-1} is a combination of the ethereal and hydroxylic C–O stretching frequencies of the carbohydrate moiety (Nakanishi, 1962).

from the spectrum of azo pigment B_6 shown in Fig. 2.

The assay for hexuronic acids gave 1.19 mol of hexuronic acid/mol of azo pigment B_4. Qualitative tests for phosphorus and sulphur were negative.

When azo pigment B_4 was incubated with β-glucuronidase, no chloroform-extractable pigment was obtained. However, reverse-phase partition chromatography revealed the presence of two water-soluble pigment fractions. The first, accounting for approx. 30–40% of the pigments, moved on the column at a rate similar to that of azo pigment B_3 (Kuenzle, 1970*b*). The second fraction (60–70%) had the same mobility as untreated azo pigment B_4. Prolonged incubation of azo pigment B_4 with larger amounts of β-glucuronidase did not alter the relative abundances of the two pigment fractions.

Azo pigment B_4 was successively incubated with β-glucuronidase and either α- or β-glucosidase. No significant amounts of chloroform-extractable pigments were generated by this double enzyme treatment.

Mild alkaline hydrolysis of azo pigment B_4 with aqueous 0.1 M-ammonia followed by solvent extraction yielded the chloroform-soluble pigment moiety of azo pigment B_4 on the one hand and the water-soluble non-pigmented compound R_4 on the other. The pigment moiety was identified as the phenylazo derivative of bilirubin by virtue of its optical, i.r. and n.m.r. spectra. These were virtually identical with the spectra of an authentic sample (Kuenzle, 1970*c*). As with azo pigment A_1 (see above), the n.m.r. spectrum differed from that of the authentic sample only inasmuch as the signals pertaining to the vinyl- and isovinyl-neoxanthobilirubinic acid isomers indicated a 2:1 molar ratio.

Compound R_4 was characterized by paper chromatography on DEAE-cellulose paper (system PC–3) as the ammonium salt of a reducing acid, since it stained brown with aniline phthalate and gave an elongated spot with R_F 0.32 (reference glucuronic acid R_F 0.28–0.38). On ordinary chromatography paper (system PC–1) compound R_4 moved with $R_{\text{Glucuronic acid}}$ 0.54, and thus proved to be more polar than glucuronic acid. It stained brown with aniline phthalate but gave no reaction with ninhydrin. Ninhydrin also failed to reveal any positive reacting material when the compound R_4 was hydrolysed with 6 M-hydrochloric acid (110°C for 16h) before paper chromatography (system PC–1).

Compound R_4 was further analysed by hydrolysis followed by trimethylsilylation and g.l.c. (systems GLC–1 and GLC–2). Enzymic hydrolysis with β-glucuronidase yielded glucuronic acid (three chromatographic peaks) and glucose (two chromatographic peaks) in approximately equal amounts, and the trimethylsilylated compounds were identified on both systems by chromatography together with authentic samples. Comparison of the peak areas with the anomer ratios of glucuronic acid and glucose from aqueous equilibrium solutions served as a further means to identify the compounds, and

good agreement with the reported values was observed [with sodium glucuronate the furanose: α-pyranose:β-pyranose proportions are 1:43:56 (Raunhardt, Schmidt & Neukom, 1967), and with glucose the α-pyranose:β-pyranose ratio is 39.8: 60.2 (Sweeley *et al.* 1963)]. After acid hydrolysis (1 M-hydrochloric acid at 100°C for 1 h) of compound R_4 and trimethylsilylation the trimethylsilyl ethers of α- and β-glucopyranose were the only compounds identified. Peaks corresponding to glucuronic acid could not be detected on g.l.c. This was attributed to the complete degradation of glucuronic acid that probably occurred during acid hydrolysis.

To gain additional information on the structure of compound R_4, the latter was treated with diazomethane and dimethyl sulphate–barium oxide, and was analysed by a combination of g.l.c. and mass spectrometry (system MS–2). With this technique compound R_4 was shown to be a mixture of three aldobiouronic acids. These were termed compounds $R_{4.1}$, $R_{4.2}$ and $R_{4.3}$ respectively. Their fully methylated methyl ester derivatives appeared on g.l.c. in approximate molar proportions 3:60:37, as determined from the respective peak areas. The three compounds were incompletely separated, causing the hepta-*O*-methyl-$R_{4.1}$ methyl ester (retention time 7.5 min) to be traced as a shoulder in the ascending portion of the hepta-*O*-methyl-$R_{4.2}$ methyl ester peak (retention time 7.9 min). In contrast, the peak of the hepta-*O*-methyl-$R_{4.3}$ methyl ester (retention time 9.0 min) was virtually uncontaminated. The mass spectra recorded from the three methylated aldobiouronic acid methyl esters are listed in Table 1. Included in Table 1 are the mass spectra of two reference compounds, i.e. hepta-*O*-methylcellobiouronic acid methyl ester

Table 1. *Mass spectra of some hepta-*O*-methylaldobiouronic acid methyl esters*

Compounds $R_{4.1}$, $R_{4.2}$ and $R_{4.3}$ were obtained as a mixture, termed compound R_4, by mild alkaline hydrolysis of azo pigment B_4. They were converted into the fully methylated methyl ester derivatives by treatment with diazomethane and dimethyl sulphate–BaO. Partial separation of the methylated compounds was achieved by g.l.c., and the mass spectra were recorded directly from the corresponding chromatographic peaks. The operational conditions (system MS–2) are described under 'Apparatus and chromatography systems' in the Experimental section. Hepta-*O*-methyl-$R_{4.1}$ methyl ester was probably contaminated with incompletely separated hepta-*O*-methyl-$R_{4.2}$ methyl ester. Hepta-*O*-methylcellobiouronic acid methyl ester and hepta-*O*-methylgentiobiouronic acid methyl ester were authentic samples used as reference compounds. The mass spectra were taken by the technique described above. Assignments were made in agreement with the fragmentation patterns as elucidated by Heyns, Grützmacher, Scharmann & Müller (1966), Kochetkov & Chizhov (1966), Kováčik, Bauer, Rosík & Kováč (1968*a*) and Kováčik, Bauer & Rosík (1968*b*). To denote the fragment ions, the symbols proposed by Kováčik *et al.* (1968*a*) were used. The letters *A* to *J* designate the various fragmentation pathways (Kochetkov & Chizhov, 1966), and the lower-case letters *a* and *b* symbolize the uronic acid and the glycose units respectively. The first lower-case letter of a symbol characterizes that unit in which the fragmentation occurred, and the second denotes the unchanged unit still attached to the fragment. Thus the symbol baA_1 refers to the first ion along the pathway *A* that arises from fragmentation of the glycose unit *b* and that is substituted by the intact uronic acid unit *a*.

	Relative intensity of signal (% of base peak)					
m/e	Hepta-*O*-methyl-$R_{4.1}$ methyl ester	Hepta-*O*-methyl-$R_{4.2}$ methyl ester	Hepta-*O*-methyl-$R_{4.3}$ methyl ester	Hepta-*O*-methylcellobiouronic acid methyl ester	Hepta-*O*-methylgentiobiouronic acid methyl ester	Assignment
468	—	—	—	—	0.02	M^+
437	0.3	0.1	0.2	—	0.02	baA_1
436	—	—	—	0.1	0.02	$M^+ - CH_3 \cdot OH$
423	—	—	0.5	—	—	baE_1
394	—	—	—	3.7	—	baB_1
379	—	0.2	4.9	—	—	$baB_1 - {}^{\bullet}CH_3$*
367	4.3	—	—	—	9.6	baD_1
333	—	0.2	0.6	—	—	abE_2
319	—	0.7	0.5	18.3	—	baF_1
303	—	—	0.6	—	—	$abE_2 - CH_2{:}O$†
301	—	—	0.6	—	—	abE_3
293	1.5	1.0	3.8	0.3	—	
292	—	—	—	—	0.6	
287	—	0.1	0.3	4.3	—	$baF_1 - CH_3 \cdot OH$
279	5.6	1.1	0.9	7.9	6.9	abJ_1

Table 1. *cont.*

	Relative intensity of signal (% of base peak)					
m/e	Hepta-*O*-methyl-$R_{4.1}$ methyl ester	Hepta-*O*-methyl-$R_{4.2}$ methyl ester	Hepta-*O*-methyl-$R_{4.3}$ methyl ester	Hepta-*O*-methylcellobiouronic acid methyl ester	Hepta-*O*-methylgentiobiouronic acid methyl ester	Assignment
233	9.1	29.8	29.1	12.0	0.4	aA_1
219	6.6	1.2	3.3	8.2	4.8	bA_1
205	4.3	6.0	6.4	—	1.0	
203	2.3	5.6	3.5	—	—	
201	5.6	4.4	7.3	84.2	8.0	aA_2
187	4.3	0.9	1.4	6.0	3.2	bA_2
173	6.1	7.1	10.0	4.7	2.1	aC_2
169	29.9	*100.0*	*100.0*	7.7	2.1	aA_3
161	9.1	1.7	2.2	4.9	1.3	bB_3
159	16.1	8.7	29.1	4.9	1.9	bC_2
157	5.2	2.1	5.2	5.2	2.9	
155	4.8	1.0	2.9	4.3	1.6	bA_3
145	12.6	7.5	12.8	8.7	3.2	
141	8.7	5.2	10.9	9.6	3.7	aC_3
131	7.8	2.1	3.3	8.1	6.9	
129	9.6	7.3	13.6	6.9	4.8	
127	6.1	1.9	3.5	7.7	3.7	bC_3
115	5.1	4.0	4.0	5.8	1.3	
111	—	1.2	—	4.0	1.7	
103	—	9.9	14.5	—	—	$CH_3 \cdot O \cdot OC \cdot CH : \overset{+}{O} \cdot CH_3$
101	*100.0*	53.2	77.4	77.0	76.8	F_1, G_1
89	8.9	6.3	9.1	9.0	9.1	
88	50.0	8.3	7.3	*100.0*	*100.0*	H_1
75	60.9	27.0	74.4	47.0	62.1	bJ_1
73	17.4	4.3	6.0	12.0	8.0	H_2
71	34.1	8.3	12.7	11.1	16.6	
59	10.9	4.8	8.2	6.0	4.3	$CH_3 \cdot O \cdot C \vdots \overset{+}{O}$
45	26.1	13.9	20.0	25.3	11.2	$CH_3 \cdot \overset{+}{O} : CH_2$

* This fragment seems not to have been reported previously. Theoretically it can occur with (1→2)-, (1→3)- and (1→4)-aldobiouronic acids. The corresponding neutral disaccharides would give rise to a similar ion of mass 365. Such an ion has been detected in the mass spectrum of octa-*O*-methylkojibiose but not in the spectrum of octa-*O*-methyllaminaribiose (C. C. Kuenzle, unpublished work).

† The mechanism leading to this fragment is analogous to the one suggested by Kochetkov & Chizhov (1966) to account for the formation of the ion E_4 from the ion E_3.

(retention time 6.7±0.2min) and hepta-*O*-methylgentiobiouronic acid methyl ester (retention time 7.4±0.2min).

Azo pigment B_5. With azo pigment B_5 the shape of the optical spectrum and the positions of the absorption maxima (270, 277, 327 and 514nm) indicated the presence of the same chromophore as occurs in the phenylazo derivative of bilirubin (Kuenzle, 1970c). The extinction coefficient at 514nm was 39.91$g^{-1}cm^{-1}$ [corrected for 12.1% inorganic impurity (probably silica) present in the sample]. From this value the molecular weight of azo pigment B_5 was calculated to be 740 (see azo pigment B_4).

The i.r. spectrum (potassium bromide) of azo pigment B_5 was virtually identical with the one shown in Fig. 2 for azo pigment B_6. An ester carbonyl band was positioned at 1730cm^{-1}.

The assay for hexuronic acids gave 1.29mol of hexuronic acid/mol of azo pigment B_5. The qualitative test for phosphorus was negative.

β-Glucuronidase hydrolysed the azo pigment B_5 quantitatively to a chloroform-extractable pigment. On columns of anhydrous sodium sulphate this pigment had the same chromatographic mobility as the phenylazo derivative of bilirubin (Kuenzle, 1970c).

After mild alkaline hydrolysis (aqueous 0.1M-ammonia) and solvent extraction, the chloroform-soluble pigment moiety of azo pigment B_5 was isolated from the organic extract, and the water-soluble non-pigmented compound R_5 was obtained

from the aqueous layer. The pigment moiety was identified as the phenylazo derivative of bilirubin (Kuenzle, 1970c) by virtue of its optical, i.r. and n.m.r. spectra. As with azo pigments A_1, A_2 and B_4 (see above), the n.m.r. spectrum revealed the presence of the vinyl- and isovinyl-neoxanthobilirubinic acid isomers in the molar ratio 2:1.

Compound R_5 was shown to contain no sulphur, and was characterized by paper chromatography as the ammonium salt of a reducing acid that proved to be more polar than glucuronic acid (see compound R_4). On DEAE-cellulose paper (system PC–3, aniline phthalate) it gave an elongated spot with R_F 0.21. On ordinary chromatography paper with systems PC–1 and PC–2 it moved with $R_{\text{Glucuronic acid}}$ 0.30 and 0.64 respectively. It stained brown with aniline phthalate, but gave no reaction with ninhydrin. Ninhydrin also failed to reveal any positive reacting material when compound R_5 was hydrolysed with 6M-hydrochloric acid (110°C for 16h) before chromatography (systems PC–1 and PC–2).

Spectroscopic analysis of compound R_5 gave little information about its structure, but established the absence of methyl, methoxyl and acetoxy groups. The i.r. spectrum (potassium bromide) showed relatively broad absorptions at 3460 (OH, NH_4^+), 2950 (CH_2, CH), 1605 (CO_2^-), 1422, 1300, 1180 to 960 [ethereal and hydroxylic C–O stretching modes (Nakanishi, 1962)], 952, 912 and 793 cm^{-1}. No absorptions were found in the methoxyl and ester carbonyl region. The n.m.r. spectrum showed two broad unresolved multiplets centred at 3.45 and 4.60p.p.m. respectively, but failed to reveal any signals indicative of methyl, methoxyl or acetoxy groups.

The acid hydrolysate (0.1M-hydrochloric acid at 100°C for 1h) of compound R_5 was trimethylsilylated and then analysed by g.l.c. (systems GLC–1 and GLC–2). When the hydrolysate was treated with aqueous 0.05M-ammonia before trimethylsilylation the trimethylsilyl derivatives of glucofuranuronic acid, α-glucopyranuronic acid and β-glucopyran-

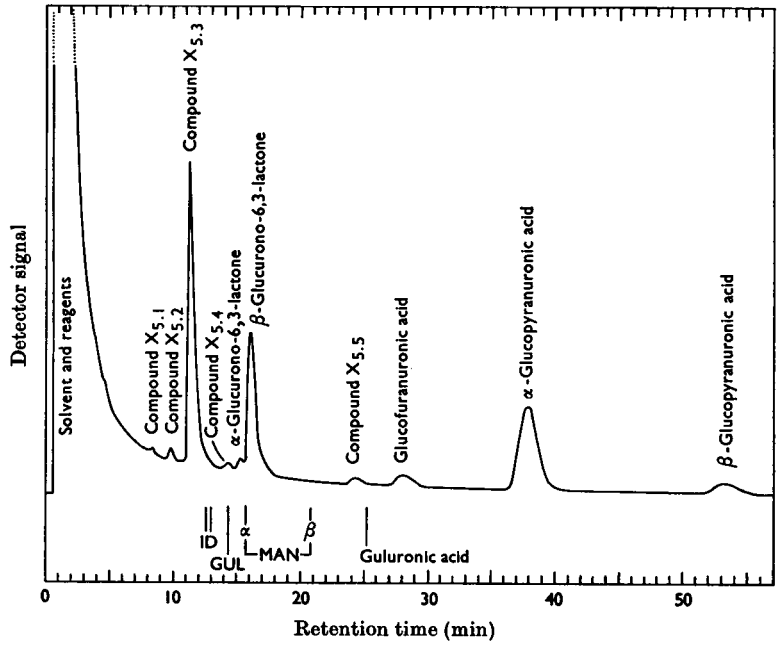

Fig. 3. G.l.c. of the trimethylsilylated hydrolysate of compound R_5. Hydrolysis was performed with 0.1M-HCl for 1h at 100°C, and the hydrolysate was not neutralized before trimethylsilylation. The conditions for g.l.c. were: glass column, 2m length, 2.7mm inside diameter; 3% SE–30 on Gas-Chrom P; T_c, 155°C; T_j, 240°C; N_2 was used as a carrier gas at a rate of 22ml/min. Glucuronolactone and glucuronic acid were identified by chromatography with authentic equilibrated samples and by mass spectrometry. The mass spectra of trimethylsilylated compounds $X_{5.1}$ to $X_{5.3}$ are given in Table 2. Compounds $X_{5.1}$ to $X_{5.4}$ are proposed to be the lactones (I) to (IV) of 3-*C*-hydroxymethyl-D-riburonic acid (Fig. 4), and compound $X_{5.5}$ is suggested to be an unspecified form of the corresponding free acid (for a more specific assignment of the lactones see the legend to Fig. 5). Vertical lines indicate the retention times of some trimethylsilylated reference compounds, i.e. iduronolactone (ID), guluronolactone (GUL), mannuronolactone (MAN) and guluronic acid.

uronic acid were the only compounds detected. They were identified on both systems by chromatography together with an authentic sample of equilibrated glucuronic acid (see compound R_4). A similar result was obtained after very mild acid hydrolysis (0.01M-hydrochloric acid at 100°C for

Table 2. *Mass spectra of some trimethylsilylated hexuronolactones*

Compounds $X_{5.1}$, $X_{5.2}$ and $X_{5.3}$ were obtained, along with some other compounds (see Fig. 3), by acid hydrolysis of compound R_5, which is the water-soluble moiety of azo pigment B_5. The hydrolysate was treated with pyridine–hexamethyldisilazane–chlorotrimethylsilane (10:2:1, by vol.), and the trimethylsilylated compounds were analysed by a combination of g.l.c. and mass spectrometry (system MS–1, see under 'Apparatus and chromatography systems' in the Experimental section). Tentative assignments of some of the fragment ions are given in Fig. 5. Authentic samples of glucuronolactone, iduronolactone, mannuronolactone and guluronolactone were trimethylsilylated and were used as reference compounds. The mass spectra were recorded by the technique described above. The spectra of trimethylsilyl-glucuronolactone and -mannuronolactone were scanned from the respective β-anomers. The spectrum of trimethylsilyliduronolactone was taken from the first of the two incompletely separated chromatographic peaks (compare Fig. 3, and Lehtonen, Kärkkäinen & Haahti, 1966).

	Relative intensity of signal (% of base peak)						
m/e	Compound $X_{5.1}$	Compound $X_{5.2}$	Compound $X_{5.3}$	Glucurono-lactone	Iduronolactone	Mannuronolactone	Guluronolactone
392	—	—	0.1	0.3	0.3	0.2	0.3
377	0.5	0.5	0.5	3.4	1.1	3.4	2.7
359	—	—	—	0.3	—	0.2	—
349	—	—	—	1.0	2.1	4.8	2.6
335	—	—	—	0.2	0.1	—*	0.2
333	—	—	—	0.6	0.1	0.3	0.3
331	—	—	—	0.2	0.2	0.4	0.2
319	—	0.3	0.1	2.2	1.2	1.1	2.3
305	—	3.5	1.6	0.2	0.1	0.2	0.4
287	—	1.0	1.0	9.0	1.3	6.3	0.7
259	1.8	3.0	2.4	5.8	3.5	10.6	4.2
258	5.5	3.4	0.7	1.0	0.1	0.9	0.1
245	—*	—*	2.9	4.6	5.5	2.0	3.0
243	37.4	22.0	5.2	4.4	2.9	8.2	2.6
230	6.0	9.8	6.9	95.2	*100.0*	89.8	*100.0*
217	1.2	4.0	4.6	14.3	15.1	15.9	15.2
215	1.4	9.1	1.8	1.9	0.9	1.0	1.9
213	1.2	1.4	1.1	2.9	—	2.2	—
204	1.2	1.5	1.3	1.9	1.7	2.2	1.9
197	—	1.5	0.7	3.6	0.2	2.1	0.4
191	1.4	1.1	1.9	2.0	1.5	2.2	1.8
189	1.0	1.6	3.4	3.2	2.9	9.0	4.0
187	1.6	2.0	2.7	0.4	0.2	—	—
173	1.4	5.0	1.2	0.6	0.4	0.6	0.9
171	3.2	3.6	1.5	1.5	0.3	1.3	0.5
169	1.8	2.7	1.2	2.7	1.1	3.2	1.3
159	5.2	—*	—*	—*	—*	—*	—*
158	1.4	22.0	68.8	—*	—*	—*	4.5
157	1.0	4.5	3.5	1.9	0.9	2.0	1.4
147	18.0	15.0	11.0	26.9	10.8	25.7	14.3
145	16.8	16.5	12.5	2.0	—*	3.4	2.6
143	3.0	5.1	1.9	2.3	2.2	2.6	2.6
133	7.7	6.5	5.0	6.9	4.9	8.6	5.3
131	6.9	8.1	4.4	4.6	2.4	4.1	3.0
129	20.6	21.0	18.7	6.1	5.9	10.0	10.9
103	3.8	8.7	6.5	6.4	5.2	6.7	7.1
75	60.6	65.0	41.7	21.4	10.0	17.1	14.7
73	*100.0*	*100.0*	*100.0*	*100.0*	70.6	*100.0*	83.3

* Actually, the spectrum contains a signal of low intensity. However, this signal is not listed since it corresponds to the isotope peak of the fragment having a mass number lower by 1 (2) mass unit(s).

0.5h) and careful neutralization to pH6 with 0.01M-sodium hydrogen carbonate. Apart from the peaks of α-glucofuranurono-6,3-lactone, β-glucofuranurono-6,3-lactone, glucofuranuronic acid, α-glucopyranuronic acid and β-glucopyranuronic acid, no other peaks were present on the chromatogram (system GLC–1). However, when the hydrolysate (0.1M-hydrochloric acid at 100°C for 1h) was trimethylsilylated without prior neutralization the chromatogram shown in Fig. 3 was obtained (system GLC–1). This showed the five peaks assigned to the various anomers of glucuronolactone and glucuronic acid. In addition, five unidentified peaks were observed, and the underlying compounds were termed compounds $X_{5.1}$ to $X_{5.5}$. Their retention times (relative to β-glucurono-6,3-lactone = 1.00) were 0.51, 0.61, 0.70, 0.88 and 1.51 respectively. The relative abundances of compounds $X_{5.1}$ to $X_{5.5}$ were calculated from the respective peak areas, and were found to be 1:3:89:1:6. The same proportions were observed in all further experiments, except for the relative amount of the compound $X_{5.5}$, which decreased to approx. 1%. This decrease went parallel with a decline in the glucuronic acid: glucuronolactone ratio.

The various compounds present in the acid hydrolysate of compound R_5 were analysed as their trimethylsilyl derivatives by a combination of g.l.c. and mass spectrometry (system MS–1). The mass spectra recorded from the peaks that had already been assigned to β-glucurono-6,3-lactone and to the three forms of glucuronic acid were found to be identical with the spectra of the authentic samples. Thus the presence of glucuronolactone and glucuronic acid was confirmed. Mass spectra were also taken of trimethylsilylated compounds $X_{5.1}$, $X_{5.2}$ and $X_{5.3}$. They are listed in Table 2 along with the mass spectra of the trimethylsilylated hexuronolactones used as reference compounds. (Mass spectra of trimethylsilylated compounds $X_{5.4}$ and $X_{5.5}$ were not taken for the following reasons. With the chromatography system used, the peak corresponding to the compound $X_{5.4}$ was located in the ascending portion of the glucuronolactone peak, and its mass spectrum would have been that of a 1:1 mixture with glucuronolactone. Compound $X_{5.5}$ was not analysed because of its low concentration, which would not have permitted the interpretation of its mass spectrum.)

The mass spectra suggested that compounds $X_{5.1}$, $X_{5.2}$ and $X_{5.3}$ were hexuronolactones. However, they differed from those of iduronolactone, mannuronolactone and guluronolactone in several aspects. The ratios of the signals at *m/e* 230 and *m/e* 243 were particularly suited to exclude the latter compounds. Iduronolactone and mannuronolactone were further ruled out by virtue of their retention times on g.l.c. (Fig. 3). The guluronolactone peak virtually coincided with that of compound $X_{5.4}$. Nevertheless guluronolactone did not qualify as a possible structure for this compound, since a peak corresponding to guluronic acid was missing on the chromatogram. The retention times of authentic guluronic acid and compound $X_{5.5}$ were sufficiently different (particularly on the column used for mass spectrometry) to rule out an identity of the latter compounds.

It was hoped that further information on the structure of compound R_5 would come from mass spectrometry of its methylated methyl ester derivative. However, all attempts to prepare this derivative were unsuccessful, since complete degradation occurred with the various methylation

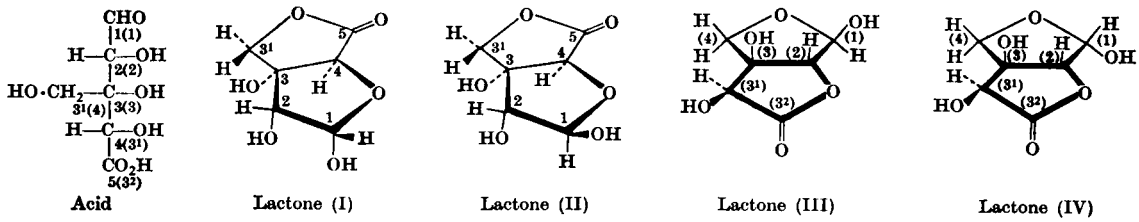

Fig. 4. 3-*C*-Hydroxymethyl-D-riburonic acid and its lactones. The open aldehyde form of the acid (Fischer projection) is shown at left, and the cyclic structures of the four lactones are given at right. Rules for the systematic naming of branched-chain sugars have been proposed by Shafizadeh (1956). According to this system the four lactones are named unambiguously as the 5,3¹-lactone of 3-*C*-hydroxymethyl-α-D-ribofuranuronic acid (lactone I), the 5,3¹-lactone of 3-*C*-hydroxymethyl-β-D-ribofuranuronic acid (lactone II), the 3²,2-lactone of 3-*C*-(**S**-carboxyhydroxymethyl)-α-L-threofuranose (lactone III) and the 3²,2-lactone of 3-*C*-(**S**-carboxyhydroxymethyl)-β-L-threofuranose (lactone IV). However, the system has the disadvantage that it assigns different numbers to identical carbon atoms of the various cyclic structures. This is illustrated with the open aldehyde form of the acid. The compound can either be named as 3-*C*-hydroxymethyl-D-riburonic acid, to which the numbering given outside the parentheses is applied, or as 3-*C*-(**S**-carboxyhydroxymethyl)-L-threose, to which the numbering given inside the parentheses is applied.

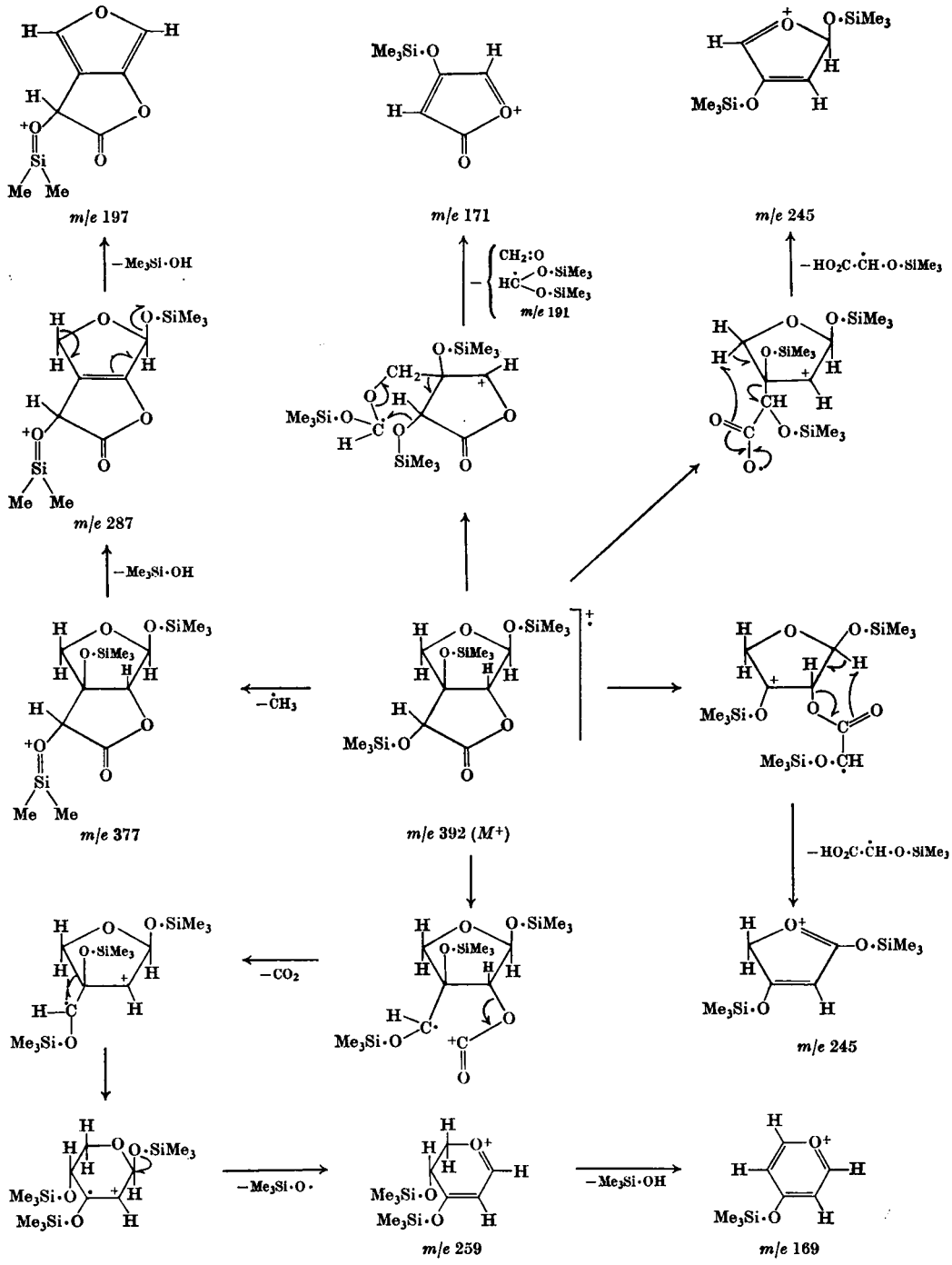

Fig. 5. Mass-spectrometric fragmentation pattern proposed for trimethylsilylated lactone (III) of 3-*C*-hydroxymethyl-D-riburonic acid (compare Fig. 4). For steric reasons the fragment of mass 245 can only form with lactones (III) and (IV) but not with lactones (I) and (II). This fragment is present in the mass spectrum of compound $X_{5.3}$ but is missing with compounds $X_{5.1}$ and $X_{5.2}$ (Table 2). Therefore the structure of either lactone (III) or lactone (IV) is tentatively assigned to the compound $X_{5.3}$, and the structures of lactones (I) and (II) are assigned to compounds $X_{5.1}$ and $X_{5.2}$. However, specific assignments cannot be made.

procedures employed (see under 'Methods' in the Experimental section). As an alternative, mass spectrometry of the fully acetylated methyl ester derivative was tried. However, the temperature needed for evaporation resulted in a thermal breakdown of the compound, and the mass spectra recorded from the thermal fragments could not be interpreted.

Optical-rotation measurements were taken from the crystals of the fully acetylated methyl ester derivative of compound R_5. The value determined was $[\alpha]_D^{21}$ +92° (*c* 0.17 in chloroform).

Azo pigment B_6. The optical spectrum of azo pigment B_6 indicated the presence of the same chromophore as occurs in the phenylazo derivative of bilirubin (Kuenzle, 1970*c*) with the shapes of the plots and the positions of the maxima (270, 277, 327 and 514nm) being virtually identical. The extinction coefficient at 514nm was 41.1 $g^{-1}cm^{-1}$ [corrected for 9.8% inorganic impurity (probably silica) present in the sample], and the molecular weight of azo pigment B_6 was calculated to be 720 (see azo pigment B_4).

The i.r. spectrum (potassium bromide) of azo pigment B_6 is shown in Fig. 2, and the assignments are given in the figure legend.

The assay for hexuronic acids gave 1.15mol of hexuronic acid/mol of azo pigment B_6. The qualitative test for phosphorus was negative.

β-Glucuronidase hydrolysed the azo pigment B_6 quantitatively to a chloroform-extractable pigment. On columns of anhydrous sodium sulphate this pigment had the same chromatographic mobility as the phenylazo derivative of bilirubin (Kuenzle, 1970*c*). In contrast, both α- and β-glucosidase failed to attack the azo pigment B_6. This was evident from the finding that incubation with either enzyme did not alter the chromatographic mobility of the pigment on reverse-phase partition chromatography columns.

After mild alkaline hydrolysis (aqueous 0.1M-ammonia) and solvent extraction, the chloroform-soluble pigment moiety of azo pigment B_6 was isolated from the organic extract, and the water-soluble non-pigmented compound R_6 was obtained from the aqueous layer. The pigment moiety was identified as the phenylazo derivative of vinyl-neoxanthobilirubinic acid (Kuenzle, 1970*c*) by virtue of its optical, i.r. and n.m.r. spectra. The experiment was repeated with a different batch of azo pigment B_6, but again no trace of the isovinyl-neoxanthobilirubinic acid isomer was detected (compare with azo pigments B_4 and B_5).

The compound R_6 was shown to contain no sulphur, and was characterized by paper chromatography as the ammonium salt of a reducing acid that proved to be more polar than glucuronic acid (see compound R_4). On DEAE-cellulose paper (system PC–3, aniline phthalate) it gave an elongated spot with R_F 0.31. On ordinary chromatography paper with systems PC–1 and PC–2 it moved with $R_{\text{Glucuronic acid}}$ 0.50 and 0.65 respectively. It stained brown (cherry-red in one instance) with aniline phthalate, but gave no reaction with ninhydrin. Ninhydrin also failed to reveal any positively reacting material when compound R_6 was hydrolysed with 6M-hydrochloric acid (110°C for 16h) before paper chromatography (systems PC–1 and PC–2).

The i.r. (potassium bromide) and n.m.r. spectra of compound R_6 were virtually identical with the spectra of compound R_5 (see above), and established the absence of methyl, methoxyl and acetoxy groups. Further, the i.r. absorptions at 3460 and 1600 cm^{-1} indicated the presence of hydroxyl and carboxylate groups respectively.

Compound R_6 was hydrolysed with 1M-hydrochloric acid (100°C for 1h), and was neutralized with aqueous 0.05M-ammonia before trimethylsilylation and g.l.c. (systems GLC–1 and GLC–2). The chromatograms showed three peaks corresponding to glucuronic acid and two peaks corresponding to glucose, and the trimethylsilylated compounds were identified on both systems by chromatography together with authentic equilibrated samples (see compounds R_4 and R_5). The glucuronic acid: glucose ratio was less than 1:1, presumably because of partial degradation of glucuronic acid during hydrolysis.

Attempts were made to hydrolyse compound R_6 with either α- or β-glucosidase. However, both enzymes failed to cleave the compound as indicated by g.l.c. of the trimethylsilylated reaction mixtures.

The repeated failure to prepare the fully methylated methyl ester derivative of compound R_6 prevented its analysis by mass spectrometry. All attempts to methylate either the methyl ester of compound R_6 or the fully acetylated methyl ester derivative of compound R_6 resulted in the complete degradation of the starting materials (see under 'Methods' in the Experimental section).

Optical-rotation measurements were taken from the crystals of the fully acetylated methyl ester derivative of compound R_6. The value determined was $[\alpha]_D^{21}$ +94° (*c* 0.31 in chloroform).

DISCUSSION

The isolation of the phenylazo derivatives of the bilirubin compounds present in human hepatic bile was reported in an accompanying paper (Kuenzle, 1970*b*). Conversion of the bilirubin compounds into the phenylazo derivatives gives protection against oxidative degradation, which is extremely rapid with the native pigments (Billing *et al.* 1957). However, azo coupling has the disadvantage of

cleaving the bilirubin compounds on both sides of the central methylene bridge, thus giving rise to a mixture composed of two unequal fragments (Kuenzle, 1970*c*). These are the phenylazo derivatives of the vinyl- and the isovinyl-neoxanthobilirubinic acid isomers (Fig. 6). Both isomers can be identified by virtue of their n.m.r. spectra (Kuenzle, 1970*c*). However, even if both isomers occur in a specific azo pigment fraction (as is the case with azo pigments A_1, A_2, B_4 and B_5), it is not possible to decide whether both isomers stem from a single bilirubin conjugate having identical substituents attached to both halves of the pigment moiety, or whether they arise from two unsymmetrically conjugated bilirubin compounds each having a specific substituent attached to different halves of the molecule. To give a better illustration of this argument, let us assume that two 'mixed' bilirubin conjugates occur, both carrying the specific substituents R_4 and R_5, but with the substituents attached to different sides of the molecule. On azo coupling, the conjugates would generate four azo pigments. One of the conjugates would give rise to the vinylneoxanthobilirubinic acid isomer carrying the substituent R_4, and to the isovinylneoxanthobilirubinic acid isomer carrying the substituent R_5. With the other conjugate the positions of the substituents would be reversed. Nevertheless only two azo pigment fractions (e.g. azo pigments B_4 and B_5) would be obtained on chromatography, since the chromatographic mobilities of the pigments are determined exclusively by the substituents. One of the fractions (azo pigment B_4) would be composed of the isomeric azo pigments both carrying the substituent R_4 but arising from different bilirubin conjugates, whereas the other (azo pigment B_5) would consist of both azo pigment isomers substituted by the radical R_5. The same result would of course be obtained with two bilirubin compounds being symmetrically conjugated with the substituents R_4 and R_5 respectively. In other words, if azo coupling is performed before the isolation of the compounds, one cannot usually decide whether both symmetrically and unsymmetrically conjugated bilirubin compounds occur in native bile.

Contrary to the above statement, unsymmetrically conjugated bilirubin compounds could be conjectured to occur if only a single azo pigment isomer (either the vinyl- or the isovinyl-neoxanthobilirubinic acid isomer) were detected in a specific azo pigment fraction. One should here remember that alkaline hydrolysis of azo pigment B_6 yielded the vinylneoxanthobilirubinic acid isomer only. Therefore the possibility of an unsymmetrically conjugated bilirubin compound arises. This conjugate would carry the substituent R_6 on the vinylneoxanthobilirubinic acid portion of the molecule and a different substituent on the isovinylneoxanthobilirubinic acid portion. This interpretation would require that an increased proportion of the isovinylneoxanthobilirubinic acid isomer should be present in one of the other azo pigment fractions. However, the contrary is the case with all the fractions analysed in this respect (the isomer ratio was 2:1 in favour of the vinylneoxanthobilirubinic acid isomer with azo pigments A_1, A_2, B_4 and B_5). On the other hand the shortcoming of the above interpretation might be overcome by the assumption of 'mixed' bilirubin conjugates carrying a substituent on their isovinylneoxanthobilirubinic acid portion that would cause the complete loss of the isomer in question. The substituent responsible for such a loss would have to be a very polar compound of relatively high molecular weight, which would render the pigment insoluble in the solvents used for isolation. The present finding of bilirubin compounds conjugated with acidic disaccharides suggests that bilirubin might also be bonded to polysaccharides. No conclusive evidence for such structures has been obtained in the present investigation. However, one is reminded of previous experiments (Kuenzle, Maier & Rüttner, 1966), performed on the diconjugated serum bilirubin fraction, that gave evidence of a molar bilirubin:hexuronic acid ratio 1:8.

One could also argue that the isovinylneoxanthobilirubinic acid isomer might be lost by preferential adsorption on denatured proteins occurring in the early stages of the isolation procedure. As with the interpretation discussed in the preceding paragraph, this explanation would be in accord with the low overall yields of the isolated azo pigments (Kuenzle, 1970*b*). However, it would not account for the discrepancy that some isovinylneoxanthobilirubinic acid isomer is present with azo pigments A_1, A_2, B_4 and B_5, whereas this isomer is completely missing with azo pigment B_6. Only if the extent of the pigment loss were also dependent on the nature of the substituents would the shortcoming of this interpretation be overcome. This again points to the possibility of high-molecular-weight conjugates of bilirubin.

An alternative explanation for the observed isomer ratio would be the natural occurrence of bilirubin XIIIα. Bilirubin XIIIα is that bilirubin isomer that possesses a symmetrical structure relative to the central methylene bridge, and, on azo coupling, gives rise to two molecules of the phenylazo derivative of vinylneoxanthobilirubinic acid. Thus the coexistence of bilirubin IXα (the normal isomer) and bilirubin XIIIα would explain both the increased proportion of the vinylneoxanthobilirubinic acid isomer occurring with azo pigments A_1, A_2, B_4 and B_5 and the exclusive occurrence of this isomer with azo pigment B_6.

However, this interpretation is untenable, since a previous investigation has established that bilirubin XIIIα does not occur in human bile (Kuenzle, 1970*c*).

Azo pigments A_1 and A_2

The structures proposed for the azo pigments A_1 and A_2 are shown in Fig. 6. They are discussed here separately under the appropriate headings.

Azo pigment A_1. This pigment was identified as the phenylazo derivative of bilirubin by virtue of its spectral properties. The elemental analysis was also in agreement with the assigned structure. However, the n.m.r. spectrum revealed that the molar ratio of the vinyl- and the isovinyl-neoxanthobilirubinic acid isomers was 2:1, contrasting with the equimolar ratio observed with an authentic sample (Kuenzle, 1970*c*). This discrepancy has already been discussed in the introductory paragraphs of the present Discussion section.

Azo pigment A_2. The optical, i.r. and n.m.r. spectra suggested that azo pigment A_2 is the phenylazo derivative of a bilirubin ester. The following arguments are in agreement with such a structure. The optical spectrum indicated the presence of the same chromophore as occurs in the phenylazo derivative of bilirubin. Theoretical arguments predict that the only functional group of the pigment which can be substituted without altering the chromophore is the carboxyl function, and in fact the optical spectra of the phenylazo derivatives of bilirubin and of bilirubin dimethyl ester are identical (Kuenzle, 1970*c*). The i.r. spectrum showed that an ester function had replaced the carboxyl group present in the phenylazo derivative of bilirubin. The n.m.r. spectrum was in agreement with this interpretation. The signal at 11.84p.p.m. accounted for approximately 2 protons. With the phenylazo derivative of bilirubin, this signal arises from 4 protons, i.e. 2 carboxyl and 2 amide protons (Kuenzle, 1970*c*). Esterification of the carboxyl groups would therefore decrease the signal area to half the value determined with the acid, as is indeed the case with azo pigment A_2.

Azo pigment A_2 was shown by n.m.r. spectroscopy to be composed of both the vinyl- and the isovinyl-neoxanthobilirubinic acid isomer. The isomer ratio was 2:1. A discussion of this unequal

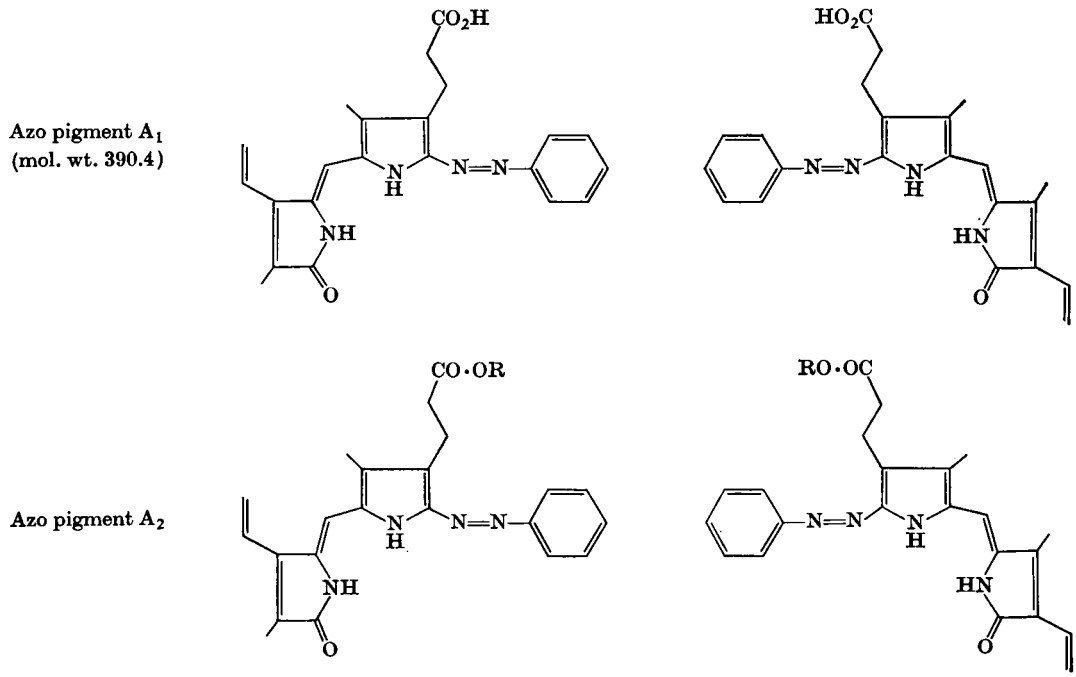

Fig. 6. Structures of azo pigments A_1 and A_2. Azo pigment A_1 is the phenylazo derivative of bilirubin, and consists of both isomers obtained on azo coupling of this bile pigment. The phenylazo derivative of the vinyl-neoxanthobilirubinic acid isomer is shown on the left and the phenylazo derivative of the isovinylneoxanthobilirubinic acid isomer on the right. Azo pigment A_2 is the phenylazo derivative of a bilirubin ester. The structure of the substituent R is unknown. However, it contains OH and/or NH_2 groups, which render the pigment more polar than azo pigment A_1.

abundance has already been given in the introductory paragraphs of the present Discussion section.

Too little information was acquired to identify the substituent present in azo pigment A_2. I.r. spectroscopy indicated that the substituent carries hydroxyl and/or amino groups, and these functions are certainly responsible for the slightly polar character of azo pigment A_2. A few n.m.r. signals were detected that were assigned to unidentified protons of the substituent, but specific assignments were not attempted. It is also uncertain whether the increased u.v. absorption of azo pigment A_2 (as compared with the phenylazo derivative of bilirubin) arises from chromophores present in the substituent or whether this reflects contamination with u.v.-absorbing impurities. The nature of the substituent therefore remains largely unknown.

Azo pigments B_1 to B_6

The structures proposed for azo pigments B_1 to B_6 are shown in Fig. 7. They are discussed separately here under the appropriate headings.

Preliminary experiments were performed with azo pigments B_1 to B_6 to screen for alkali-stable and alkali-labile pigments. Pigments were classified depending on whether or not they were stable to very mild alkaline hydrolysis (0.05M-sodium hydroxide at 20°C for 0.5h). Among the six compounds tested, azo pigment B_1 was the only alkali-stable pigment detected. Alkali-stable pigments have been reported to account for up to 50% of the total bilirubin conjugates (Billing *et al.* 1957; Isselbacher & McCarthy, 1959; Gregory & Watson, 1962*a*; Vegas, 1963; Noir, Groszman & DeWalz, 1966; Kuenzle *et al.* 1966). However, the proportion of azo pigment B_1 amounts to approx. 0.4% only (Kuenzle, 1970*b*). Moreover, the pigment seems to be missing from most bile specimens (Kuenzle, 1970*b*). This discrepancy is not completely understood, but is tentatively attributed to the relative purities of the pigments tested. Previous assays for alkali-stable conjugates have been performed on either native bile (Billing *et al.* 1957) or serum (Gregory & Watson, 1962*a*), or on relatively crude azo pigments (Isselbacher & McCarthy, 1959; Vegas, 1963; Noir *et al.* 1966; Kuenzle *et al.* 1966). It is possible that proteins and lipids of bile exert some kind of a protecting action on the alkali-labile pigments, thus simulating an increased proportion of alkali-stable conjugates.

It has been repeatedly claimed that the alkali-stable bilirubin fraction of human bile is bilirubin sulphate (Isselbacher & McCarthy, 1959); Tenhunen, 1965; Noir *et al.* 1966). Although such a compound has been identified in rats (Isselbacher & McCarthy, 1959; Schoenfield, Bollman & Hoffman, 1962; Gregory & Watson, 1962*b*), some investigators have denied its occurrence in humans (Gregory & Watson, 1962*b*; Vegas, 1963; Weber & Schalm, 1965). The present investigation gave no evidence for a sulphate compound. Azo pigment B_1 was the most likely candidate for such a structure, since it resisted mild alkaline hydrolysis. However, a sulphate structure could be ruled out by virtue of the negative test for sulphur. Sulphur was also shown to be absent from azo pigments B_4, B_5 and B_6. Sulphur determinations were not carried out with azo pigments B_2 and B_3. However, it seems unlikely that the latter compounds are sulphates. Three arguments against such structures can be forwarded; both pigments were alkali-labile, i.r. absorptions due to C–O–S vibrations of acid sulphates (Lloyd, Tudball & Dodgson, 1961) were missing in the range 820–850 cm^{-1} and the chromatographic mobilities indicated that both pigments were markedly less polar than the phenylazo derivative of a synthetic bilirubin sulphate. The synthetic sulphate derivative proved to be even more polar than azo pigment B_6, and therefore did not qualify as a candidate for a naturally occurring compound. Vegas (1963) has come to the same conclusion by using ion-exchange chromatography to differentiate between naturally occurring bilirubin conjugates and synthetic bilirubin sulphate.

Azo pigment B_1. The structure of an alkyl glycoside of a hydrated bilirubin derivative was tentatively assigned to azo pigment B_1 (Fig. 7). Such a structure would account for both the alkali-stability and the light-absorption properties of this compound. The optical spectrum of azo pigment B_1 was virtually identical with the one obtained from the phenylazo derivative of mesobilirubin (Kuenzle, 1970*c*) except for a slight hypsochromic shift of the major absorptions. The glycosyl nature of the substituent was suggested by the similarity of the i.r. spectra recorded from azo pigment B_1 on the one hand and azo pigments B_4 to B_6 on the other. As discussed below, azo pigments B_4 to B_6 are glycosides, and the conclusions about the structure of azo pigment B_1 are therefore reasonable. However, the nature of the glycosyl radical remains unknown. Glucuronic acid is an unlikely candidate, since the pigment is not cleaved by β-glucuronidase, and phosphate and sulphate esters of carbohydrates are ruled out by the negative tests for phosphorus and sulphur. The possible occurrence of a second substituent, which might be attached to the carboxyl group of the pigment moiety, also remains open to discussion. This uncertainty is due to the low resolution of the i.r. bands, which prevented the identification of specific carbonyl frequencies.

Azo pigments B_2 and B_3. Both pigments are proposed to be acyl glycosides of the phenylazo derivative of bilirubin. This conclusion derives mainly from the alkali-lability of the pigments,

since acyl glycosides are known to be readily cleaved under the conditions applied. The absorption maxima at 514nm, as observed in the optical spectra of both compounds, were in agreement with the proposed ester structures (for a discussion see under 'Azo pigment A_2'), and the similarity of the i.r. spectra of azo pigments B_2 to B_6 was consistent with the presence of glycosyl moieties (for a discussion see under 'Azo pigment B_1'). Lack of material prevented the identification of the glycosyl radicals.

Azo pigment B_4. This pigment is not a homogeneous compound, but is a mixture consisting of the phenylazo derivatives of three acyl aldobiouronides of bilirubin. The three compounds were tentatively identified as an acyl 6-*O*-hexopyranosyluronic acid-hexoside, an acyl 4-*O*-hexofuranosyluronic acid-D-glucoside and an acyl 4-*O*-β-D-glucofuranosyluronic acid-D-glucoside.

The pigment moiety of azo pigment B_4 was isolated after alkaline hydrolysis of this compound. It was identified by spectroscopic methods as the phenylazo derivative of bilirubin. The identification was not impeded by the evidence for a 2:1 ratio of the vinyl- and the isovinyl-neoxanthobilirubinic acid isomers, although an equimolar ratio should be expected to occur with any symmetrical conjugate of bilirubin IXα (for a discussion see the introductory paragraphs of the present Discussion section).

An acyl glycoside structure was strongly suggested by the alkali-lability of azo pigment B_4 (for a discussion see under 'Azo pigments B_2 and B_3'). The optical spectrum was consistent with this structure, since it indicated the presence of the same chromophore as occurs in the phenylazo derivative of bilirubin (for a discussion see under 'Azo pigment A_2'). The i.r. absorption at 1733 cm^{-1} confirmed the proposed ester group.

Although the naphtharesorcinol reaction indicated that azo pigment B_4 contained 1 mol. prop. of hexuronic acid, the first hypothesis of a glucuronide structure was quickly abandoned. This was mainly because neither the molecular weight, determined to be 810 (this value is high by approx. 11.2% as compared with the nominal value of 728.7; the high value is attributed to the presence of impurities arising from the chromatographic support; this conclusion derives from the finding that contamination of the pigments increased in reversed proportion to their concentrations on the columns used for isolation; thus contamination of the pigments also increased in the order azo pigments B_5 and B_6<B_4<B_2 and B_3; with azo pigments B_5 and B_6 the molecular weights determined agreed with the nominal values within ±0.4 and ±1.2% respectively), nor the failure of β-glucuronidase to cleave the compound at the ester function were in agreement with such a structure. Rather,

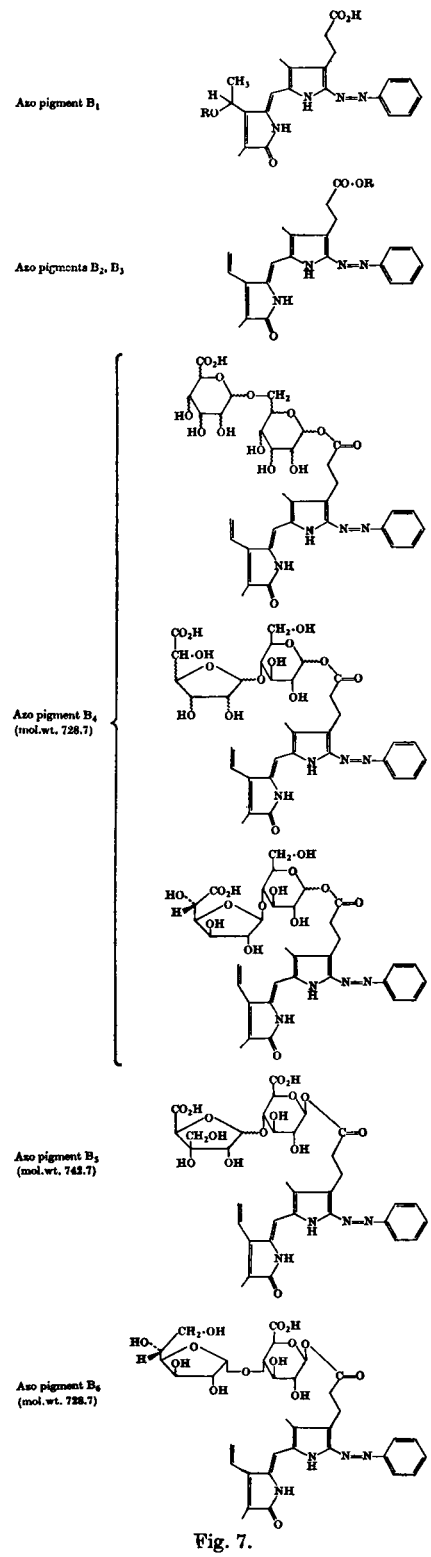

Fig. 7.

they suggested a more complex structure with a hexuronic acid involved.

In agreement with this interpretation were the results obtained by paper chromatography of compound R_4 (the substituent cleaved from the pigment moiety by mild alkaline hydrolysis). These suggested the presence of a reducing sugar of greater polarity than glucuronic acid. Further, the occurrence of a free acid grouping was established by the observation that compound R_4 was retarded on anion-exchange cellulose.

With the failure to detect phosphate, sulphate or amino acids as conceivable components of compound R_4, there remained the possibility of a disaccharide structure to account for a substituted hexuronic acid. Indeed, acid hydrolysis of compound R_4 yielded glucose, which was identified by trimethylsilylation and g.l.c. However, the expected hexuronic acid could not be detected on the chromatograms probably because of extensive degradation of this compound. On the other hand, when compound R_4 was subjected to the action of β-glucuronidase, glucose and glucuronic acid were obtained in approximately equimolar proportions. This indicated the presence of an acidic disaccharide composed of glucose and glucuronic acid. The sensitivity of compound R_4 towards β-glucuronidase established, not only the sequence of the two carbohydrate units as corresponding to that of an aldobiouronic acid (glucuronic acid linked glycosidically to glucose), but also the β-D-configuration of the glucosyluronic acid radical. In contrast, the ring size of the glucosyluronic acid radical could not be derived from this information, since both β-D-glucopyranosiduronic acids and β-D-glucofuranosiduronic acids are known to be cleaved by β-glucuronidase (Kato, Yoshida & Tsukamoto, 1964*a*,*b*). Thus the structure of an *O*-β-D-glucosyluronic acid-D-glucose was assigned to compound R_4, with the D-configuration of the glucose unit being derived from the well-documented fact that L-glucose does not occur in humans.

Actually, the proposed structure fits only one of the three aldobiouronic acids that make up compound R_4, but at the time the above structural assignment was made compound R_4 was still assumed to be homogeneous. Subsequent experiments showed that only part of azo pigment B_4 could be converted into a less polar pigment (the acyl glucoside of the phenylazo derivative of bilirubin) by the action of β-glucuronidase. The glucuronidase-sensitive component of azo pigment B_4 amounted to approx. 30–40% of the total pigments present in this fraction. An explanation for this finding only suggested itself when the results of the mass-spectrometric analysis of methylated compound R_4 became available.

When the fully methylated methyl ester derivative of compound R_4 was analysed by a combination of g.l.c. and mass spectrometry, it became apparent that compound R_4 consisted of a mixture of three aldobiouronic acids. These occurred in the molar proportions 3:60:37, and were termed compounds $R_{4.1}$, $R_{4.2}$ and $R_{4.3}$ respectively. The fragmentation patterns of the three compounds were in agreement with hexosyluronic acid-hexose structures (Heyns *et al.* 1966; Kochetkov & Chizhov, 1966; Kováčik *et al.* 1968*a*).

Compound $R_{4.1}$ was tentatively identified as a 6-*O*-hexopyranosyluronic acid-hexose by virtue of the characteristic peak at *m/e* 367, which occurs with (1→6)aldobiouronic acids only (Heyns *et al.* 1966; Kochetkov & Chizhov, 1966; Kováčik *et al.* 1968*a*). The (1→6)linkage was further confirmed by the absence of a signal *m/e* 319. The hexuronic acid unit was suggested to be a pyranoside, since

Fig. 7. Structures of azo pigments B_1 to B_6. The pigment moiety of each azo pigment is shown as the vinylneoxanthobilirubinic acid isomer (see Fig. 6), and, indeed, this is the only isomer found with azo pigment B_6. In contrast, azo pigments B_4 and B_5 also contain the isovinylneoxanthobilirubinic acid isomer. With azo pigments B_1 to B_3 no experimental evidence has been obtained as to which isomer occurs. The pigment moiety of azo pigment B_1 is shown to carry a carboxyl group, although an ester or amide function may actually be present. The substituents R of azo pigments B_1 to B_3 presumably are glycosyl radicals. Azo pigment B_4 is a mixture of three acyl aldobiouronides. The first and the second of these are suggested to have the structures of an acyl 6-*O*-hexopyranosyluronic acid-hexopyranoside and of an acyl 4-*O*-hexofuranosyluronic acid-D-glucopyranoside respectively, with the hexuronic acid units being either α-D-glucuronic acid or diastereomers of glucuronic acid. The third aldobiouronide is proposed to be an acyl 4-*O*-β-D-glucofuranosyluronic acid-D-glucopyranoside. The ring sizes of the hexuronic acid units have been established by mass spectrometry. In contrast, the hexose units are arbitrarily drawn as the pyranosides. Azo pigment B_5 is an acyl hexuronosylhexuronide, and is shown as the acyl 4-*O*-(3-*C*-hydroxymethyl-D-ribofuranosyluronic acid)-β-D-glucopyranosiduronic acid. Actually, the branched-chain hexuronic acid unit may be present as the 3-*C*-(S-carboxyhydroxymethyl)-L-threofuranosyl radical with its structure corresponding to the free acid of either the lactone (III) or the lactone (IV), both shown in Fig. 4. Further, neither the absolute configuration of the branched-chain hexuronic acid nor the (1→4)-disaccharide linkage have been ascertained, and the glucuronic acid unit is arbitrarily drawn as the pyranoside. Azo pigment B_6 is an acyl pseudoaldobiouronide, and is shown as the acyl 4-*O*-α-D-glucofuranosyl-β-D-glucopyranosiduronic acid. However, neither the (1→4)-linkage nor the ring sizes of the carbohydrate units have been conclusively established.

signals indicative of a hexofuranosyluronic acid radical were missing (see the following paragraphs). The hepta-*O*-methyl-$R_{4.1}$ methyl ester and the hepta-*O*-methylgentiobiouronic acid methyl ester virtually coincided on g.l.c. Nevertheless they were recognized as individual compounds, since their mass spectra were sufficiently different to rule out a possible identity. Thus compound $R_{4.1}$ did not rate as a candidate for the β-D-glucosyluronic acid-D-glucose structure indicated by a previous experiment (see above).

Compound $R_{4.2}$ was characterized as a 4-*O*-hexofuranosyluronic acid-hexose. The (1→4)-linkage was indicated by the ions occurring at *m/e* 319 and 161. Whereas the fragment of mass 319 is typical of (1→4)- and (1→2)-linkages, the ion at *m/e* 161 cannot form with (1→2)-aldobiouronic acids (Kochetkov & Chizhov, 1966; Kováčik *et al.* 1968*a*). The furanoside structure of the hexuronic acid unit was suggested by the low intensity of the ion at *m/e* 88 (Heyns *et al.* 1966; Kochetkov & Chizhov, 1966), and was confirmed by the occurrence of the ions at *m/e* 333 and 103. The fragment of mass 333 arises from the molecular ion by elimination both of $CH_3 \cdot OH$ and of the uronic ester side chain $(CH_3 \cdot O \cdot OC \cdot \dot{C}H \cdot O \cdot CH_3)$. Alternatively, the uronic ester side chain produces the ion at *m/e* 103 $(CH_3 \cdot O \cdot OC \cdot CH{:}\overset{+}{O} \cdot CH_3)$.

Compound $R_{4.3}$ was identified as a 4-*O*-hexofuranosyluronic acid-hexose by the reasoning given in the preceding paragraph. The ions of mass 303 and 301 further confirmed the furanoside structure of the hexuronic acid unit. These ions probably arise from the fragment of mass 333 by elimination of $CH_2{:}O$ and $CH_3 \cdot OH$ respectively.

The question arose as to which of the three aldobiouronic acids might possibly have the structure of a β-D-glucosyluronic acid-D-glucose. Since compound $R_{4.1}$ did not rate as a candidate for such a structure (see above), the problem was reduced to deciding between compounds $R_{4.2}$ and $R_{4.3}$. The question was tentatively answered by relating the proportion of the β-glucuronidase-sensitive component of azo pigment B_4 to the relative abundances of compounds $R_{4.2}$ and $R_{4.3}$. With a proportion of 30–40% of azo pigment B_4 being cleaved by β-glucuronidase, and with the hepta-*O*-$R_{4.3}$ methyl ester accounting for 37% of the total aldobiouronic acid derivatives, the structure of a 4-*O*-β-D-glucofuranosyluronic acid-D-glucose was assigned to compound $R_{4.3}$. However, the evidence for this assignment is far from conclusive, since, conceivably, the relative yields of the fully methylated aldobiouronic acid methyl esters might not reflect the actual proportions of the parent compounds.

Thus, with the structure of the compound $R_{4.3}$ being tentatively established, there remained the problem of the identity of the carbohydrate units present in compounds $R_{4.1}$ and $R_{4.2}$. G.l.c. of the trimethylsilylated acid hydrolysate of compound R_4 had indicated that no other sugar than glucose occurred as the hexose unit. Therefore it was concluded that glucose had been released, not only from compound $R_{4.3}$, but also from compound $R_{4.2}$. It seems unlikely that any other hexose might have been degraded to a greater extent than glucose. Also, there is no need to account for different rates of hydrolysis, since both compounds contain a hexofuranosyluronic acid radical, and in general furanosides are readily cleaved by acids. In contrast, it is doubtful whether glucose also occurs with compound $R_{4.1}$. The hexuronic acid in this compound is a pyranoside, and aldobiouronic acids possessing this structural unit are known to be difficult to cleave by acids. Further, the small proportion of this compound would probably have prevented detection of the hexose unit even if hydrolysis had occurred.

The hexuronic acids present in compounds $R_{4.1}$ and $R_{4.2}$ also remain unidentified, since they seem to have undergone extensive degradation during acid hydrolysis. Alternatively, degradation may have been caused by the subsequent alkaline treatment. The latter possibility might point to the occurrence of L-iduronic acid which is known to be particularly sensitive to alkali. On the other hand, the glucuronic acid released from compound $R_{4.3}$ was also completely degraded. Therefore the hexuronic acid units of compounds $R_{4.1}$ and $R_{4.2}$ might be conceived to occur as the α-D-glucosyluronic acid radicals, which would also be resistant to the action of β-glucuronidase.

No experiments were devised to elucidate the ring sizes of the hexose units present in either of the three aldobiouronic acids. In contrast, attempts were made to elucidate the stereochemistry of the glucosidic linkage by which compound $R_{4.3}$ is attached to the pigment moiety. However, the glucosidic bond seemed to be resistant to both an α- and a β-glucosidase. It is uncertain whether the resistance of the bond was caused by the general failure of the enzymes to cleave acyl glucosides, or whether the enzymes were inhibited by either the pigment moiety or the dimethyl sulphoxide added to solubilize the substrate. Still another possibility might be the occurrence of a glucofuranoside structure, which would probably not be attacked by the stereospecific enzymes used.

Azo pigment B_5. This pigment was tentatively identified as an acyl 4-*O*-(3-*C*-hydroxymethyl-D-ribofuranosyluronic acid)-β-D-glucopyranosiduronic acid, with the acyl radical being that of the phenylazo derivative of bilirubin.

Many of the analyses performed with azo pigment B_5 and its products of alkaline hydrolysis (the pigment moiety and compound R_5) gave results similar to those obtained with azo pigment B_4. This suggested related structures for the two pigments. However, the finding that β-glucuronidase cleaved azo pigment B_5 at the acyl glycoside linkage gave evidence of a reversed sequence of the carbohydrate units. The presence of glucuronic acid was confirmed by g.l.c. of the trimethylsilylated acid hydrolysate of compound R_5. In contrast, the second carbohydrate unit was degraded by the alkaline treatment used to convert glucuronolactone into the ammonium salt of the corresponding acid. The sensitivity of this second unit proved to be so pronounced that the compound did not even resist degradation under neutral conditions.

Only when compound R_5 was hydrolysed with dilute acid and neutralization was omitted did the trimethylsilyl derivatives of the second carbohydrate unit appear on g.l.c. The chromatograms indicated that the sugar existed in five forms, and these were designated compounds $X_{5.1}$ to $X_{5.5}$. Compound $X_{5.3}$ was the most prominent of these, and accounted for approx. 89% of the various forms present. The mass spectra recorded from the trimethylsilyl derivatives of compounds $X_{5.1}$, $X_{5.2}$ and $X_{5.3}$ suggested that these compounds were hexuronolactones. The mass spectra and the chromatographic retention times were sufficiently different from those of trimethylsilylated glucuronolactone, iduronolactone, mannuronolactone and guluronolactone to rule out a possible identity with any of these compounds (Table 2 and Fig. 3). Since the latter hexuronolactones are the only ones known to form from straight-chain hexuronic acids, a branched-chain structure remained as the only alternative to account for the experimental data.

Of all the conceivable hexuronic acid isomers, only two needed further consideration as possible structures for the isolated compound. This conclusion derived from the finding that the underlying acid gave rise to at least three lactones (compounds $X_{5.1}$ to $X_{5.3}$). For steric reasons most hexuronic acid isomers cannot form more than two lactones (the lactones are stable only if they consist of two condensed five-membered rings, i.e. if a furanose ring and a γ-lactone ring share a common carbon–carbon bond). Exceptions are the 3-*C*-hydroxymethylriburonic acid, which affords the four lactones shown in Fig. 4, and the 2-*C*-carboxyhydroxymethylthreose, which theoretically might give three lactones (the α-anomer and the β-anomer of a furanose structure where the γ-lactone forms between the carboxyl group on the side chain and the hydroxyl group on C-3 of the sugar backbone, and the α-anomer of a furanose structure where the γ-lactone forms between the carboxyl group on the side chain and the hydroxyl group on C-1 of the sugar backbone).

The mass spectra recorded from trimethylsilylated compounds $X_{5.1}$ to $X_{5.3}$ were in agreement with the fragmentation pattern proposed for the lactones of 3-*C*-hydroxymethylriburonic acid (Table 2 and Fig. 5). In contrast, the mass spectra were not compatible with the structure of the 2-*C*-carboxyhydroxymethylthreose, which was ruled out by the following arguments (see Fig. 5). The fragment of mass 169 appears in the spectra of trimethylsilylated compounds $X_{5.1}$, $X_{5.2}$ and $X_{5.3}$. This contrasts with the theoretical prediction that only one of the lactones of 2-*C*-carboxyhydroxymethylthreose (the structure with the lactone ring that involves the hydroxyl group on C-1 of the sugar backbone) would give this particular fragment. The two other lactones would not yield this ion since the fragmentation would only proceed to the ion of mass 259, in which the substituents would not be adequately positioned to allow for the elimination of trimethylsilanol. Similarly with this compound the configurations of any of its lactones would be unfavourable for the rearrangement leading to the ion at *m/e* 171 [elimination of $(CH_3)_3Si\cdot O\cdot \dot{C}H\cdot O\cdot Si(CH_3)_3$ is not possible]. Further evidence against this structure was derived from the finding that the fragment of mass 245 occurs exclusively with compound $X_{5.3}$. In contrast, this fragment would be expected to arise from two of the lactones of 2-*C*-carboxyhydroxymethylthreose (from both anomers of the structure with the lactone ring that involves the hydroxyl group on C-3 of the sugar backbone; with the third lactone the hydrogen atom on C-3 of the sugar backbone would be unfavourably oriented to allow for its rearrangement to the carbonyl oxygen of the carboxyl group).

The proposed mechanism of fragmentation (Fig. 5) not only supports the structure of the 3-*C*-hydroxymethylriburonic acid but also allows the various lactone configurations to be assigned to compounds $X_{5.1}$ to $X_{5.3}$ (for the configurations of the four lactones of 3-*C*-hydroxymethylriburonic acid, see Fig. 4). The assignments are based on the finding that the ion at *m/e* 245 occurs with compound $X_{5.3}$ but is missing with compounds $X_{5.1}$ and $X_{5.2}$ (Table 2). It is suggested that this ion arises by cleavage of the lactone ring followed by an elimination that proceeds analogously to a McLafferty rearrangement (Fig. 5). The structural requirements for this rearrangement are only met by lactones (III) and (IV) but not by lactones (I) and (II), which cannot eliminate $HO_2C\cdot\dot{C}H\cdot O\cdot Si(CH_3)_3$. Therefore, the structure of either lactone (III) or lactone (IV) is tentatively assigned to compound $X_{5.3}$, and the structures of lactones (I) and (II) are assigned to compounds $X_{5.1}$ and

$X_{5.2}$. However, specific assignments cannot be made. It is also suggested that compounds $X_{5.4}$ and $X_{5.5}$ are respectively the fourth lactone and an unspecified form of the free acid. This conclusion derives from the finding that the relative proportions of compounds $X_{5.1}$ to $X_{5.4}$ were constant in various experiments, whereas the relative amount of compound $X_{5.5}$ diminished parallel with the observed decrease of the glucuronic acid: glucuronolactone ratio.

In Fig. 7 the branched-chain hexuronic acid radical of azo pigment B_5 is arbitrarily shown as the 3-*C*-hydroxymethyl-D-ribofuranosyluronic acid. Actually it has not been possible to decide which of the four structures of 3-*C*-hydroxymethylriburonic acid occurs. [These would correspond to lactones (I) to (IV) shown in Fig. 4. However, the branched-chain hexuronic acid unit present in azo pigment B_5 does not occur as a lactone but as a free acid. This is indicated by the absence of a lactone band from the i.r. spectrum of azo pigment B_5.] Corresponding conclusions were prevented by the failure to prepare the fully methylated methyl ester derivative of compound R_5. Neither has the absolute configuration of the branched-chain hexuronic acid been established. This was prevented by the lack of appropriate reference compounds that would have permitted the optical rotation to be correlated with either the D- or the L-configuration.

No conclusive evidence has been obtained as to the type of linkage that bonds the two carbohydrate units. A (1→4)-linkage was suggested by the sensitivity of compound R_5 to the alkaline conditions prevailing during methylation. This observation would be in agreement with the known alkali-sensitivity of 4-*O*-substituted uronic acids and particularly of the corresponding methyl esters (Whistler & BeMiller, 1958). However, in view of the finding that the hepta-*O*-acetylpseudomaltobiouronic acid methyl ester could be readily methylated (C. C. Kuenzle, unpublished work), the proposed type of linkage does not withstand criticism. Therefore the occurrence of either a (1→2)- or a (1→3)-linkage might be proposed with almost equal probability. Another unidentified structural feature of azo pigment B_5 concerns the ring size of the glucuronic acid unit. The lability of the glucuronoside linkage towards the action of β-glucuronidase does not allow one to distinguish between the pyranoside and furanoside structures, since both are known to be cleaved by the enzyme (Kato *et al.* 1964*a*,*b*).

A word should be added about the naphtharesorcinol reaction of 3-*C*-hydroxymethylriburonic acid. The reaction performed with azo pigment B_5 indicated the presence of 1.29 mol of hexuronic acid/mol of azo pigment. In view of the finding that the molar ratios observed with azo pigments B_4 and B_6 were 1.19:1 and 1.15:1 respectively, with 1 mol. prop. being accounted for by the glucuronic acid unit, it is suggested that an excess value of 0.15–0.19 mol.prop. arises from blue pigments produced from the azo pigment moiety during the naphtharesorcinol reaction. With this information it can be concluded that 3-*C*-hydroxymethylriburonic acid gives a naphtharesorcinol reaction that only amounts to approx. 10% of that observed with glucuronic acid.

Azo pigment B_6. This pigment was tentatively identified as the acyl 4-*O*-α-D-glucofuranosyl-β-D-glucopyranosiduronic acid, with the acyl radical being that of the phenylazo derivative of vinylneoxanthobilirubinic acid.

Most of the experiments performed with either azo pigment B_6 or compound R_6 (the substituent cleaved from the pigment by mild alkaline hydrolysis) gave results similar to those obtained with azo pigment B_5 and compound R_5 respectively. However, these pigments were found to differ from each other in two fundamental respects. The first concerns the pigment moiety, which for azo pigment B_6, was shown to be the phenylazo derivative of vinylneoxanthobilirubinic acid. This finding has already been discussed in the introductory paragraphs of the present Discussion section. The second concerns the carbohydrate moiety, which in azo pigment B_6 proved to be a pseudoaldobiouronic acid composed of glucose and glucuronic acid.

The proposed sequence of the sugar units, which corresponds to that of a pseudoaldobiouronic acid (glucose linked glycosidically to glucuronic acid), was indicated by the sensitivity of the acyl glycoside bond to the action of β-glucuronidase. This also established the β-D-configuration of the glucuronic acid unit, but did not distinguish between the glucopyranosyluronic acid and the glucofuranosyluronic acid structure (for a discussion see under-'Azo pigment B_5').

The glucose unit is shown in Fig. 7 as the α-D-glucofuranoside. This structure was suggested when Hudson's isorotation rules (Hudson, 1909; Bose & Chatterjee, 1958) were applied to the molecular rotation of the fully acetylated methyl ester derivative of compound R_6. As shown in Table 3, the molecular rotation is in good agreement with the value computed for a derivative of *O*-α-D-glucofuranosyl-α-D-glucopyranuronic acid. However, it also falls within the limits set by an *O*-α-D-glucopyranosyl-α-D-glucopyranuronic acid structure on the one hand and an *O*-α-D-glucopyranosyl-β-D-glucopyranuronic acid structure on the other. Thus the molecular rotation establishes the α-D-configuration of the glucose unit, but leaves the proposed furanoside structure open to criticism. Evidence for a glucofuranosyl structure might also

Table 3. *Molecular rotations computed for some pseudoaldobiouronic acid derivatives*

Compound	$[M]_D$
Hepta-*O*-acetyl methyl ester derivative of compound R_6	+624°*
O-α-D-Glucofuranosyl-α-D-glucopyranuronic acid methyl ester, 2,3,5,6-tetra-*O*-methyl-, triacetate	+628°†
O-α-D-Glucofuranosyl-β-D-glucopyranuronic acid methyl ester, 2,3,5,6-tetra-*O*-methyl-, triacetate	+300°†
O-α-D-Glucopyranosyl-α-D-glucopyranuronic acid methyl ester, hepta-acetate	+758°‡
O-α-D-Glucopyranosyl-β-D-glucopyranuronic acid methyl ester, hepta-acetate	+431°‡
O-β-D-Glucopyranosyl-α-D-glucopyranuronic acid methyl ester, hepta-acetate	+376°‡
O-β-D-Glucopyranosyl-β-D-glucopyranuronic acid methyl ester, hepta-acetate	+49°‡

* Computed from $[\alpha]_D^{21}$ +94° (*c* 0.31 in chloroform) and mol. wt. 664.

† Computed from the specific rotations of methyl 2,3,5,6-tetra-*O*-methyl-α-D-glucofuranoside and of either the α- or the β-D-glucopyranuronic acid methyl ester tetra-acetate. The specific rotations have been taken from Bose & Chatterjee (1958).

‡ Computed from the specific rotations of α-D-glucopyranose penta-acetate, β-D-glucopyranose penta-acetate, α-D-glucopyranuronic acid methyl ester tetra-acetate and β-D-glucopyranuronic acid methyl ester tetra-acetate respectively (Bose & Chatterjee, 1958).

come from the finding that compound R_6 was not hydrolysed by an α-glucosidase. The failure of the enzyme to cleave the α-glucosidic bond present would be in accord with the proposed glucofuranoside structure, since a furanoside would probably not be attacked owing to the stereospecificity of the enzyme. This interpretation is supported by the fact that the operational conditions were optimum for the enzyme used.

There remains to be discussed the question as to what type of linkage occurs between the glucose and the glucuronic acid units. A (1→4) linkage is tentatively proposed to account for the observation that compound R_6 was degraded during methylation. The evidence for this type of linkage is, of course, far from conclusive (for a discussion see under 'Azo pigment B_5'), and either a (1→2)- or a (1→3)-linkage may actually be present. However, the structure of a 4-*O*-α-D-glucopyranosyl-D-glucuronic acid (pseudomaltobiouronic acid) is unlikely to qualify, since this compound has been shown to be readily converted into the corresponding hepta-*O*-methyl methyl ester derivative (C. C. Kuenzle, unpublished work). This observation further supports the above conclusions as to a furanoside structure of the glucose unit.

General conclusions

The present investigation shows that the major bilirubin conjugates of human bile occur as three acyl aldobiouronides (azo pigment B_4), one acyl hexuronosylhexuronide (azo pigment B_5) and one acyl pseudoaldobiouronide (azo pigment B_6). A simple glucuronide, previously reported to be the major bilirubin conjugate (Talafant, 1956; Billing *et al.* 1957; Schmid, 1957), has not been detected.

Many types of conjugates are known to occur with normal metabolites and with drugs (for a review of the literature see Williams, 1967), but conjugation with acidic disaccharides has not been reported previously. Aldobiouronic acids are rather common compounds. They are generally obtained from naturally occurring acidic polysaccharides (for a review of the literature see Whistler & Rowell, 1966) or by oxidation of suitably protected neutral disaccharides (Jayme & Demming, 1960; Lindberg & Selleby, 1960). In contrast, pseudoaldobiouronic acids are rare substances. The only known pseudoaldobiouronic acids of natural origin are the 2-*O*-α-D-glucopyranosyl-D-glucuronic acid isolated from a mould (Barker, Gómez-Sánchez & Stacey, 1959) and the 3-*O*-β-D-glucopyranosylglucuronic acid obtained by enzymic hydrolysis of pneumococcal type III polysaccharide (Torriani & Pappenheimer, 1962). So far, only four pseudoaldobiouronic acids have been prepared by chemical syntheses. These are 4-*O*-β-D-glucopyranosyl-D-glucuronic acid (Johansson, Lindberg & Theander, 1963; Roy & Timell, 1968), 4-*O*-α-D-glucopyranosyl-D-glucuronic acid (Hirasaka & Matsunaga, 1965), 2-*O*-α-D-glucopyranosyl-D-galacturonic acid (Šipoš & Bauer,

1968) and 2-*O*-β-D-glucopyranosyl-D-galacturonic acid (Šipoš & Bauer, 1968). Hexuronosylhexuronic acids can be prepared by oxidation of suitably protected neutral disaccharides (Hirasaka, 1963), but to date have not been detected in Nature. Of particular note is the present finding of a branched-chain hexuronic acid occurring as one of the components of the isolated hexuronosylhexuronide. Branched-chain sugars are widely distributed among plants and microorganisms (Shafizadeh, 1956; Berry, 1963; Grisebach, 1967; Okuda *et al.* 1967, 1968; Gustine & Kindel, 1969), but previously have not been isolated from animals nor humans.

The present findings raise the question whether metabolites other than bilirubin and drugs reported to be excreted as glucuronides might not actually be conjugated with acidic disaccharides similar to those attached to bilirubin. This possibility exists with all compounds that have been identified as glucuronides exclusively by virtue of their susceptibility to attack by β-glucuronidase. However, hydrolysis by β-glucuronidase is not a sufficient argument for a glucuronide structure. This is evident from the present finding that, contrary to current concepts (Levvy & Conchie, 1966), at least some *O*-substituted β-glucuronides (such as the hexuronosylglucuronide and the glucosylglucuronide of bilirubin) are cleaved by the enzyme.

Bilirubin and its conjugates are commonly regarded as meaningless degradation products of haemoglobin, and no significance is attributed to their high concentrations in bile. With this concept, the importance of the intestinal reabsorption of bilirubin (enterohepatic circulation) (Lester & Schmid, 1963*a*,*b*) must remain obscure. However, the solution to this problem might come from speculations as to what functions the bilirubin conjugates might possibly have. From this aspect the structures proposed above for the major bilirubin conjugates must be powerful anionic detergents. Clearly the two acidic disaccharides linked to the propionic acid side chains of bilirubin make up the highly polar part of the molecule, whereas the bilirubin moiety has strong affinities to non-polar solvents. Thus the structures suggest that the detergent activities of the bilirubin conjugates might be even higher than those of most bile acids.

I express my gratitude to Professor C. H. Eugster for letting me pursue my own research project in his laboratory, and to Professor J. R. Rüttner for his interest in the present investigation. I also thank my colleagues who have contributed to this work by scanning the n.m.r. spectra (Dr C. Mayer), the mass spectra (Dr A. Chang Sin-Ren) and the i.r. spectra (Mr H. Frohofer). I also express my thanks to Professor R. Gitzelmann for providing the g.l.c. facilities and for his gift of a valuable enzyme, to Professor K. Grob for preparing the capillary column and to many investigators for their generous gifts of precious reference compounds. I am indebted to Dr C. L. Green for linguistic advice and for reviewing the manuscript. This study was supported by a research grant from Fritz Hoffmann–La Roche Stiftung, Basle, Switzerland.

REFERENCES

Barker, S. A., Gómez-Sánchez, A. & Stacey, M. (1959). *J. chem. Soc.* p. 3264.
Berry, M. (1963). *Q. Rev. chem. Soc.* **17**, 343.
Billing, B. H., Cole, P. G. & Lathe, G. H. (1957). *Biochem. J.* **65**, 774.
Bose, A. K. & Chatterjee, B. G. (1958). *J. org. Chem.* **23**, 1425.
Cummins, D. L., Gitzelmann, R., Lindenmann, J. & Semenza, G. (1968). *Biochim. biophys. Acta*, **160**, 396.
Diner, U. E., Sweet, F. & Brown, R. K. (1966). *Can. J. Chem.* **44**, 1591.
Edington, R. A. & Percival, E. (1955). *J. chem. Soc.* p. 3554.
Feigl, F. (1960). *Tüpfelanalyse*, Band II, p. 66. Frankfurt: Akademische Verlagsgesellschaft.
Fishman, W. H. & Green, S. (1955). *J. biol. Chem.* **215**, 527.
Fishman, W. H., Springer, B. & Brunetti, R. (1948). *J. biol. Chem.* **173**, 449.
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.
Grisebach, H. (1967). *Biosynthetic Patterns in Microorganisms and Higher Plants*, p. 66. New York: John Wiley and Sons Inc.
Grob, K. (1968). *Helv. chim. Acta*, **51**, 718.
Gustine, D. L. & Kindel, P. K. (1969). *J. biol. Chem.* **244**, 1382.
Helferich, B. & Berger, A. (1957). *Chem. Ber.* **90**, 2492.
Heyns, K., Grützmacher, H. F., Scharmann, H. & Müller, D. (1966). *Fortschr. chem. Forsch.* **5**, 448.
Hirasaka, Y. (1963). *J. pharm. Soc. Japan*, **83**. 971; cited by Marsh, C. A. (1966). In *Glucuronic Acid*, p. 3. Ed. by Dutton, G. J. New York: Academic Press Inc.
Hirasaka, Y. & Matsunaga, I. (1965). *Chem. pharm. Bull., Tokyo*, **13**, 176.
Hudson, C. S. (1909). *J. Am. chem. Soc.* **31**, 66.
Isselbacher, K. J. & McCarthy, E. (1959). *J. clin. Invest.* **38**, 645.
Jayme, G. & Demming, W. (1960). *Chem. Ber.* **93**, 356.
Johansson, I., Lindberg, B. & Theander, O. (1963). *Acta chem. scand* **17**, 2019.
Kato, K., Yoshida, K. & Tsukamoto, H. (1964*a*). *Chem. pharm. Bull., Tokyo*, **12**, 664.
Kato, K., Yoshida, K. & Tsukamoto, H. (1964*b*). *Chem. pharm. Bull., Tokyo*, **12**, 670.
Kochetkov, N. K. & Chizhov, O. S. (1966). *Adv. Carbohyd. Chem.* **21**, 39.
Kolínská, J. & Semenza, G. (1967). *Biochim. biophys. Acta*, **146**, 181.
Kováčik, V., Bauer, Š. & Rosík, J. (1968*b*). *Carbohyd. Res.* **8**, 291.
Kováčik, V., Bauer, Š., Rosík, J. & Kováč, P. (1968*a*). *Carbohyd. Res.* **8**, 282.
Kuenzle, C. C. (1970*a*). *Chimia*, **24**, 199.

Kuenzle, C. C. (1970*b*). *Biochem. J.* **119**, 387.
Kuenzle, C. C. (1970*c*). *Biochem. J.* **119**, 395.
Kuenzle, C. C., Maier, C. & Rüttner, J. R. (1966). *J. Lab. clin. Med.* **67**, 294.
Kuhn, R. & Trischmann, H. (1963). *Chem. Ber.* **96**, 284.
Lehtonen, A., Kärkkäinen, J. & Haahti, E. (1966). *J. Chromat.* **24**, 179.
Lester, R. & Schmid, R. (1963*a*). *J. clin. Invest.* **42**, 736.
Lester, R. & Schmid, R. (1963*b*). *New Engl. J. Med.* **269**, 178.
Levvy, G. A. & Conchie, J. (1966). In *Glucuronic Acid*, p. 320. Ed. by Dutton, G. J. New York: Academic Press Inc.
Lindberg, B. & Selleby, L. (1960). *Acta chem. scand.* **14**, 1051.
Lloyd, A. G., Tudball, N. & Dodgson, K. S. (1961). *Biochim. biophys. Acta*, **52**, 413.
Masamune, H. & Satake, M. (1959). *Tohoku J. exp. Med.* **69**, 167.
Nakanishi, K. (1962). *Infrared Absorption Spectroscopy—Practical*, p. 34. San Francisco: Holden-Day Inc.
Noir, B. A., Groszman, R. J. & DeWalz, A. T. (1966). *Biochim. biophys. Acta*, **117**, 297.
Okuda, S., Suzuki, N. & Suzuki, S. (1967). *J. biol. Chem.* **242**, 958.
Okuda, S., Suzuki, N. & Suzuki, S. (1968). *J. biol. Chem.* **243**, 6353.
Raunhardt, O., Schmidt, H. W. H. & Neukom, H. (1967). *Helv. chim. Acta*, **50**, 1267.
Roy, N. & Timell, T. E. (1968). *Carbohyd. Res.* **7**, 17.
Schmid, R. (1957). *J. biol. Chem.* **229**, 881.
Schoenfield, L. J., Bollman, J. L. & Hoffman, H. N. (1962). *J. clin. Invest.* **41**, 133.
Semenza, G., Curtius, C. H., Kolínská, J. & Müller, M. (1967). *Biochim. biophys. Acta*, **146**, 196.
Shafizadeh, F. (1956). *Adv. Carbohyd. Chem.* **11**, 263.
Šipoš, P. & Bauer, Š. (1968). *Carbohyd. Res.* **6**, 494.
Sweeley, C. C., Bentley, R., Makita, M. & Wells, W. W. (1963). *J. Am. chem. Soc.* **85**, 2497.
Talafant, E. (1956). *Nature, Lond.*, **178**, 312.
Tenhunen, R. (1965). *Annls Med. exp. Biol. Fenn.* **43**, (Suppl.), 6.
Torriani, A. & Pappenheimer, A. M. (1962). *J. biol. Chem.* **237**, 3.
Vegas, F. R. (1963). *Analyt. Biochem.* **5**, 465.
Wagner, H. (1957). *Mikrochim. Acta*, p. 19.
Wallenfels, K., Bechtler, G., Kuhn, R., Trischmann, H. & Egge, H. (1963). *Angew. Chem.* **75**, 1014.
Weber, A. P. & Schalm, L. (1965). *Acta med. scand.* **177**, 519.
Weissmann, B. & Meyer, K. (1954). *J. Am. chem. Soc.* **76**, 1753.
Whistler, R. L. & BeMiller, J. N. (1958). *Adv. Carbohyd. Chem.* **13**, 289.
Whistler, R. L. & Rowell, R. M. (1966). In *Glucuronic Acid*, p. 137. Ed. by Dutton, G. J. New York: Academic Press Inc.
Williams, R. T. (1967). In *Biogenesis of Natural Compounds* 2nd ed., p. 589. Ed. by Bernfeld, P. Oxford: Pergamon Press Ltd.