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Mass-Spectrometric Study of the Azopigments Obtained from Bile Pigments with Diazotized Ethyl Anthranilate

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The structures of some azopigments obtained by diazotization of conjugated and unconjugated bile pigments with diazotized ethyl anthranilate were studied by mass spectrometry. The α_0-azopigments derived from rat bile and human bile were shown to be identical (t.l.c. and mass spectra) with azobilirubin derived from unconjugated bilirubin. The presence of two methyl vinyl isomers (Ia) and (Ib) in equal amounts was shown by t.l.c. and mass spectrometry. The structure of the δ-azopigment derived from rat bile was studied by two methods: (*a*) ammonolysis gave rise to an amide having a $CH_2 \cdot CH_2 \cdot CO \cdot NH_2$ side chain as shown by its mass spectrum; (*b*) the mass spectrum of a trimethylsilyl derivative of the δ-azopigment methyl ester confirmed the ester to be a β-D-monoglucuronide ester of azobilirubin I.

Diazotization of bilirubin and conjugated bilirubin with *p*-diazobenzenesulphonic acid gives rise to cleavage at the central methylene bridge of the tetrapyrrole molecule with formation of 2 molecules of dipyrrole azopigments. Kinetic studies (Overbeek, Vink & Deenstra, 1955; Brodersen, 1960; Lucassen, 1961) and analysis of the reaction products (Fischer & Haberland, 1935; Overbeek *et al.* 1955) supported this conclusion. The reaction course has been analysed in detail by Lucassen (1961).

From bilirubin, azopigments of type A (azobilirubin) are formed with diazotized sulphanilic acid and diazotized aniline; from conjugated bilirubin an azobilirubin β-D-monoglucuronide, known as azopigment B, was isolated (Billing, Cole & Lathe, 1957; Schmid, 1957). The purity of these azopigment B preparations has been questioned (Heirwegh, Van Hees, Leroy, Van Roy & Jansen, 1970). The ready hydrolysis of azopigment B with dilute alkali (Billing *et al.* 1957) and the formation of a new pigment, assumed to be a hydroxamate, with hydroxylamine (Schachter, 1957) were taken as evidence for an ester linkage of glucuronic acid with the carboxyl group of azobilirubin. However, as adequate model compounds are lacking, additional evidence is required to exclude the possibility of a glucuronidic ether linkage with the enolized lactam group of azobilirubin.

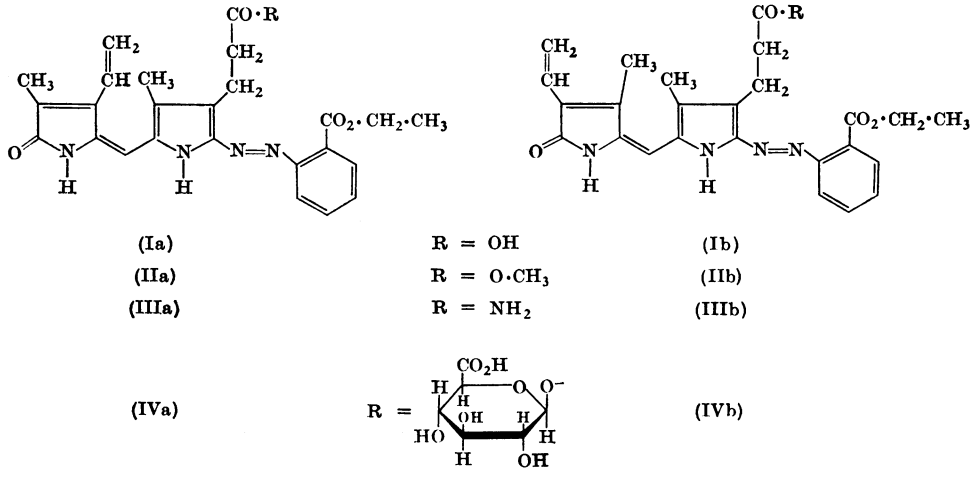
In the present paper it is shown that diazotization of bile pigments with diazotized ethyl anthranilate leads to dipyrrole compounds as in the reaction with *p*-diazobenzenesulphonic acid. The structures of two azopigments, α_0 and δ, are investigated by mass spectrometry. Evidence is presented that the δ-azopigment corresponds to the azobilirubin β-D-monoglucuronide (IV, Scheme 1), having the glucuronidic linkage on the pyrrole side-chain carboxyl group.

RESULTS AND DISCUSSION

Replacement of *p*-diazobenzenesulphonic acid by diazotized ethyl anthranilate in the diazotization of bile pigments (Heirwegh *et al.* 1970) leads to less polar azopigments, which can more conveniently be purified and characterized. The α_0- and δ-azopigments obtained from conjugated bilirubin (rat bile and human bile) were separated and further purified by t.l.c. on silica gel.

The α_0-azopigment (rat bile and human bile) shows identical chromatographic properties with those of the azopigments produced by diazotization of unconjugated bilirubin. By repetitive development, t.l.c. in each case reveals two closely moving components present in about equal amounts. By using azobilirubin sufficient material could be separated for determination of their mass spectra. Both components show a molecular ion at *m/e* 462 and a nearly identical fragmentation pattern. This evidence agrees with the existence of two isomers (Ia and Ib, Scheme 1) differing only in the position of the methyl and vinyl groups, as expected from the asymmetrical structure of bilirubin.

Short treatment of each acid isomer with diazomethane leads to methylation of the carboxyl group only (M^+ 476). In the solvent system chloroform–benzene (1:1, v/v) the methyl ester of each acid isomer separates into two components (both



(Ia) R = OH (Ib)

(IIa) R = O·CH$_3$ (IIb)

(IIIa) R = NH$_2$ (IIIb)

(IVa) R = (IVb)

Scheme 1.

showing a molecular ion at *m/e* 476), the relative amounts of which varied, however, as much as 0:1 to 1:0 in several separate methylation experiments. The latter observation could be explained by: (*a*) the CO·NH function in pyrrole ring A giving rise to keto–enol isomers; (*b*) syn–anti isomerism caused by the N=N bond; (*c*) a change in the conjugated double bond system, e.g. a shift of a double bond in pyrrole ring B. The presence of an ion *m/e* 389 (loss of $CH_2 \cdot CH_2 \cdot CO \cdot O \cdot CH_3$), which only appears in the mass spectra of the more polar methyl esters, suggests a structural difference in the vicinity of the propionic acid side chain, in agreement with possibilities (*b*) and (*c*). The present evidence does not allow, however, a safe structural assignment to the latter isomers.

Ammonolysis of the δ-azopigment leads to the isolation of a major derivative which has a molecular ion (*m/e* 461) and fragmentation pattern consistent with the amide structures (IIIa) and (IIIb) (Scheme 1). These structures follow from a comparison with the mass spectrum (Fig. 1*a*) of the α_0-azopigment (mixture of Ia and Ib), and with the mass spectra (Figs. 1*b* and 1*c*) of two methylated fractions, derived from the mixture of α_0-isomers by a short treatment with diazomethane. The latter fractions correspond to a t.l.c. separation of 'double bond or syn–anti isomers' (see above) but still consist of a mixture of methyl vinyl isomers.

In all spectra allylic cleavage of the side chain occurs with formation of an ion *m/e* 403 (loss of $CH_2 \cdot CO \cdot R$; R=OH, O·CH$_3$ or NH$_2$). Elimination of the whole side chain is involved in the formation of the ion *m/e* 389 present for (I), (III) and the more polar methyl ester fraction. Loss of CO·R is observed only for (III) (*m/e* 417, $M^+ - CO \cdot NH_2$).

The formation of abundant ions at *m/e* 298 (I), 312 (methyl esters), 297 (III) and *m/e* 165 (I, methyl esters and III) requires cleavage of the N=N bond with, for the first series of ions, migration of one hydrogen and for *m/e* 165, migration of two hydrogens to the anthranilic part of the molecule.

The complete hydrolysis of the δ-azopigment derived from rat bile with β-glucuronidase, inhibited by the the addition of saccharo-(1→4)-lactone (Heirwegh *et al.* 1970), indicates that the β-hydroxy group at C-1 of glucuronic acid is involved in bond formation. The mass spectrum of the δ-azopigment, after treatment with diazomethane and bistrimethylsilylacetamide, shows molecular ions at *m/e* 796 and *m/e* 868. These correspond with an azobilirubin conjugated with a hexuronic acid, in which one methyl group and two (M^+ 796) or three (M^+ 868) trimethylsilyl groups have been introduced.

The introduction of one methyl group in the α_0- and δ-azopigments by reaction with diazomethane indicates that in both compounds only one carboxyl group is available for methylation. For azobilirubin (I) clearly the side-chain carboxyl group is involved. For the δ-azopigment either the side-chain carboxyl group or the carboxyl group of glucuronic acid must be free. In fact, as mentioned above, studies with β-glucuronidase show that glucuronic acid is bound to (I) with its β-hydroxy group at C-1 and not with its carboxyl group. Accordingly, structure (IV) has to be assigned to the δ-azopigment.

The presence of a glucuronidic ester linkage in the δ-azopigment is also indicated by the structure of the amide (III) isolated after treatment of the δ-azopigment with ammonia vapour. Indeed, the mass spectrum of this compound unequivocally

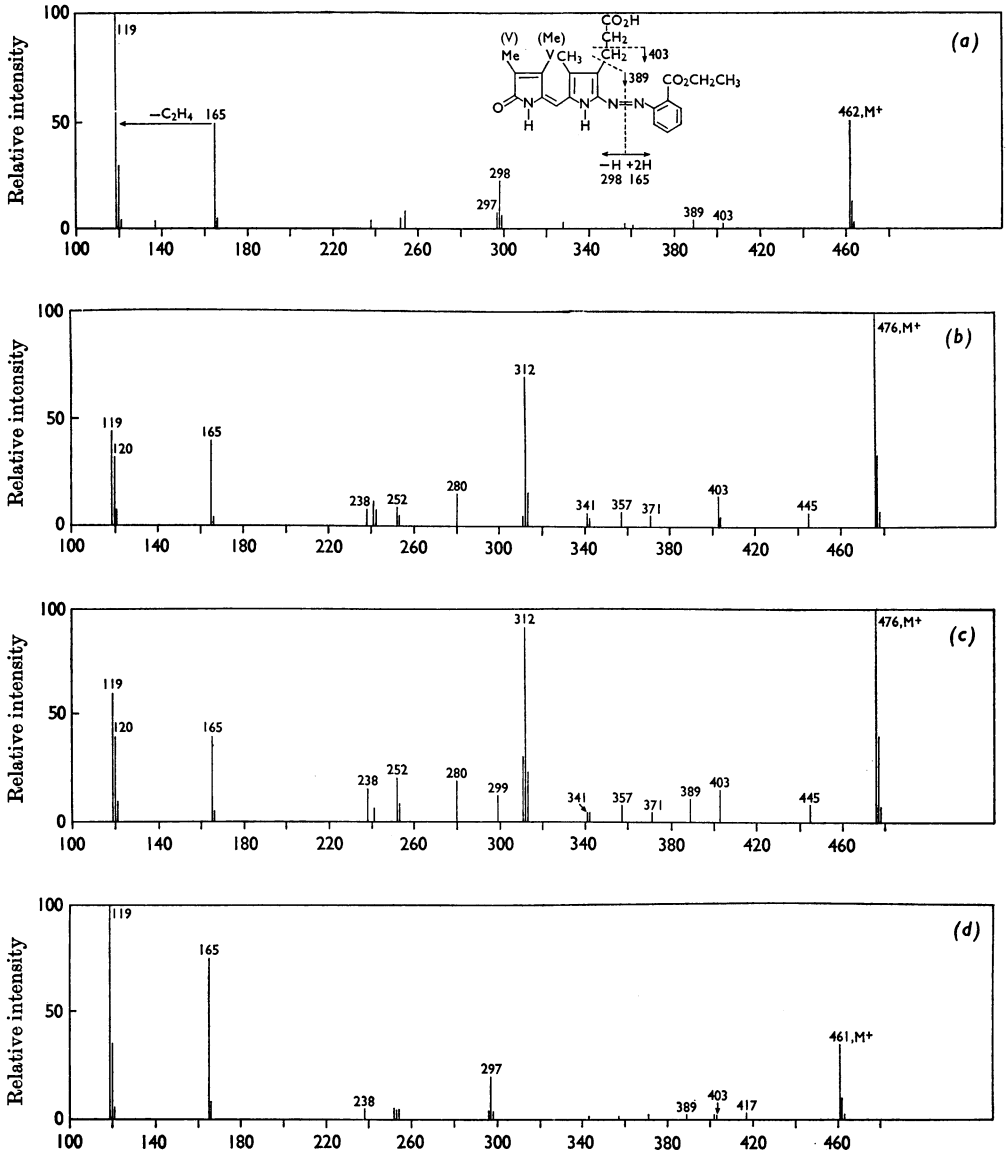


Fig. 1. Mass spectra of (*a*) α_0-azopigment (mixture of Ia and Ib, Scheme 1); (*b*) and (*c*) less polar and more polar (t.l.c.) methyl ester fraction of α_0 (both fractions are a mixture of methyl vinyl isomers); (*d*) amide derivative of δ-azopigment (III, Scheme 1).

proves the presence of a $CH_2 \cdot CH_2 \cdot CO \cdot NH_2$ side chain. The original existence of an ester linkage is indicated in this case by the observation that, under the same experimental conditions, the amide (III) is not formed from the α_0-azopigment.

The isolation of the same amide (III) from other conjugates as demonstrated by t.l.c. (Heirwegh *et al.* 1970) indicates that they also contain a similar ester linkage.

EXPERIMENTAL

Preparation of azopigments. Azopigments α_0 and δ, obtained from rat bile, α_0 derived from human bile and azobilirubin derived from unconjugated bilirubin were purified as described by Heirwegh *et al.* (1970). Separation of the azobilirubin and the α_0-azopigment isomers was performed by t.l.c. on pre-coated Merck F254 silica gel plates (E. Merck A.-G., Darmstadt, Germany). Seven

developments in the solvent system chloroform–ethyl acetate (1:1, v/v) in each case revealed the same pair of pigments present in about equal amounts.

Elementary analysis of azobilirubin was performed (Found: C, 64.7; H, 5.8; N, 11.9; O, 17.3. Calc. for $C_{25}H_{26}N_4O_5$: C, 64.9; H, 5.7; N, 12.1; O, 17.3%).

Methylation of α_0-azopigments and azobilirubin. The pigments were dissolved in chloroform and treated with ethereal diazomethane during a few seconds, followed by evaporation of the solvent *in vacuo*. The same methylation procedure was applied on each azobilirubin isomer separated as described above. Two developments (t.l.c. on Merck F254 silica gel) in the solvent system chloroform–benzene (1:1, v/v) showed the presence of two red azobilirubin derivatives in variable intensity (0:1 to 1:0 in several separate methylation experiments performed on samples of the purified azobilirubin isomers). The derivatives obtained with diazomethane were chromatographically indistinguishable when the isolated azobilirubin isomers (Ia or Ib) or the initial mixture were examined (no separation of the methyl vinyl isomers occurs for the methyl esters).

Mass spectra (Figs. 1*b* and 1*c*) were determined on the two methylated t.l.c. fractions derived from an α_0-sample (rat bile). No separation of methyl vinyl isomers was performed before methylation. The mass spectra were distinguishable by the presence of an ion at m/e 389 for the more polar fraction.

When the α_0-azopigment was treated with diazomethane for more than 1 min an appreciable quantity of a much more polar pigment was formed (M^+ 490).

Trimethylsilyl derivative of δ-azopigment methyl ester. The methyl ester of the δ-azopigment (rat bile) was prepared by addition of ethereal diazomethane to a solution of the azopigment in chloroform and a minimal amount of methanol. After purification by preparative t.l.c. (Merck F254 silica gel) in the solvent system chloroform–methanol (17:3, v/v), this methyl ester (1–2 mg) was treated with 1 ml of chloroform and 5 drops of bistrimethylsilylacetamide (Sweeley, Bentley, Makita & Wells, 1963) during 1 h. After evaporation of chloroform *in vacuo*, a sample was introduced in the mass spectrometer through the direct-introduction lock, where the excess of reagent was evaporated.

Preparation of amide (III) from δ-azopigment. Purified δ-azopigment, applied as a band to thin-layer plates (DC-Alufolien silica gel; E. Merck A.-G.) was kept overnight at room temperature in a closed chromatographic tank saturated with ammonia vapour. After evaporation of ammonia in the air the plates were developed with chloroform. The least polar band was removed, transferred to a small column and eluted with chloroform.

Mass spectra. The samples were run through the direct introduction lock of an MS 12 AEI mass spectrometer (Manchester, U.K.) at an ionizing voltage of 70 eV, 8 kV accelerating voltage and 500 μA ionizing current. The temperature of the ion source was raised slowly from 180°C to 220°C.

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REFERENCES

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

Brodersen, R. (1960). *J. clin. Lab. Invest.* **12**, 25.

Fischer, H. & Haberland, H. W. (1935). *Hoppe-Seyler's Z. physiol. Chem.* **232**, 235.

Heirwegh, K. P. M., Van Hees, G. P., Leroy, P., Van Roy, F. P. & Jansen, F. H. (1970). *Biochem. J.* **120**, 877.

Lucassen, J. (1961). Doctoral Thesis: University of Utrecht.

Overbeek, J. Th. G., Vink, C. L. J. & Deenstra, H. (1955). *Recl Trav. chim. Pays-Bas Belg.* **74**, 85.

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

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

Sweeley, C. C., Bentley, R., Makita, M. & Wells, W. W. (1963). *J. Am. chem. Soc.* **85**, 2497.