Analyses of azopigments obtained from the delta fraction of bilirubin from mammalian plasma (mammalian biliprotein)

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Azopigments were obtained from the delta fraction of bilirubin (mammalian biliprotein) in cholestatic sera of men, rats and guinea pigs by diazo reaction with diazotized *p*-iodoaniline and analysed by t.l.c. Delta bilirubin of men and rats generated both unconjugated and glucuronide-conjugated azodipyrroles, whereas that of guinea pigs, in which the predominant form of conjugated bilirubin in serum was bilirubin monoglucuronide, generated only unconjugated azodipyrrole. We further analysed the azopigments by reversed-phase h.p.l.c. to distinguish their endovinyl and exovinyl isomers. The results indicated (a) that covalent binding of bilirubin to protein occurs exclusively on the conjugated dipyrrolic (either endovinyl or exovinyl) half of the parent conjugated bilirubin, (b) that both bilirubin monoglucuronide and bilirubin diglucuronide generate delta bilirubin, the latter yielding a 'conjugated' form of delta bilirubin that preserves the glucuronic acid moiety on the dipyrrolic half not bound covalently to protein, and (c) that therefore at least four forms of delta bilirubin exist in jaundiced sera of men and rats.

INTRODUCTION

Delta bilirubin is a fraction of bilirubin that appears in plasma of patients with conjugated hyperbilirubinaemia and is thought to be a bilirubin–albumin covalent complex, i.e. a kind of biliprotein. This fraction of serum bilirubin was first reported by Kuenzle and co-workers in 1966 [1,2], and Lauff and his colleagues confirmed its existence by h.p.l.c. in 1981 [3,4]. Its clinical significance has been the subject of several subsequent studies [5–8].

It is generally accepted that delta bilirubin is formed from non-enzymic combination of conjugated bilirubin and albumin in jaundiced plasma [9,10]. Although the exact structure of this bilirubin–albumin complex has not been fully clarified, recent studies suggest that formation of an amide bond between one of the propionic acid side chains of bilirubin and a nucleophilic site on protein, probably a lysine residue, occurs [5,9].

Delta bilirubin is essentially 'direct-reacting' on diazo reaction, and the resulting azopigments were reported to be mainly unconjugated ones [4]. It was thus assumed that delta bilirubin originated from monoconjugated bilirubin, in which the conjugating moiety was cleaved during the formation of the covalent bond [1,8]. Several points raise doubts as to the validity of this assumption. (a) The yields of the formation of bilirubin–albumin covalent complexes from bilirubin monoglucuronide (BMG) and bilirubin diglucuronide (BDG) *in vitro* were nearly equal [9]. (b) *In vivo*, both guinea pigs and rats produced delta bilirubin after bile-duct ligation [9], although the predominant form of conjugated bilirubin in bile [11] and serum (K. Kamisaka, personal communication) of the former species is BMG and that of the latter is BDG. (c) The efficiency of the diazo reaction performed in the presence of protein is questionable.

In the present study we utilized a more reliable method of diazo reaction to identify the form(s) of parent tetrapyrrole in delta bilirubin obtained from sera of cholestatic men, rats and guinea pigs.

EXPERIMENTAL

Materials

5-Amino[4-¹⁴C]laevulinic acid hydrochloride (specific radioactivity 53 mCi/mmol) was purchased from Amersham International. Essentially globulin- and free-fatty-acid-free rat serum albumin was purchased from Sigma Chemical Co. In the preparation of delta bilirubin, we utilized an Amicon model 202 ultrafiltration system with YM-10 membrane. T.l.c. plates (Kieselgel 60, 5 cm × 20 cm) were purchased from Merck. In the h.p.l.c. studies, we utilized systems SP8700 and SP8750 with Toyo Soda visible-region and ultraviolet spectrophotometers. H.p.l.c. columns ODS-120A and G3000SW were purchased from the same company. Radioactivity was quantified in an Aloka 3300 liquid-scintillation counter. The other chemicals used were of analytical grade.

Preparation of delta bilirubin

The common bile ducts of male Wistar rats (*n* = 4, body weight 200–250 g) and male Hartley guinea pigs (*n* = 4, body weight 200–250 g) were ligated under light pentobarbital anaesthesia. After 3 days blood samples were collected from the abdominal aortas, and serum was separated. Human icteric sera were obtained from patients with obstructive jaundice (*n* = 3). The sera were washed four times with caffeine/benzoate reagent [4] in the ultrafiltration system, and the albumin fraction was separated by salting out with (NH₄)₂SO₄ as described by

Abbreviations used: BMG, bilirubin monoglucuronide; BDG, bilirubin diglucuronide.

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Lauff *et al.* [4]. The resulting mixture of bilirubin–albumin covalent complex with intrinsic albumin was used as ‘delta bilirubin’, since no reliable method for further purification is available.

Preparation of bile

Rat bile was collected through indwelling PE-10 cannulas inserted into the bile ducts of male Wistar rats. Human bile was sampled freshly from patients with obstructive jaundice through percutaneous bile-drainage tubes. Samples were used immediately or were stored at -40°C and used within a week.

H.p.l.c. analysis of serum delta bilirubin

To determine delta bilirubin in jaundiced sera, 10 μl of unprocessed serum was gel-filtered on a G3000SW h.p.l.c. column in 6M-urea in 50 mM-sodium phosphate buffer, pH 7.0. Absorbances at 280 nm and 450 nm were recorded simultaneously. Under these conditions non-covalent binding between bilirubin and serum protein is cleaved and only delta bilirubin is co-eluted with albumin [12].

H.p.l.c. analysis of conjugated bilirubin

To analyse the composition of conjugated bilirubin in jaundiced sera, we eluted deproteinized sera on an ODS-120A column according to the method described by Uesugi *et al.* [11]. In this method only bilirubins dissociable from protein were detected, and bilirubin–albumin covalent complex was lost during the deproteinization. The mobile phase consisted of 50 mM-sodium citrate buffer (pH 5.0)/methanol/acetonitrile (8:9:3, by vol.). The absorbance at 450 nm was recorded.

Diazo reaction of delta bilirubin

Samples (10 mg) of delta bilirubin obtained from jaundiced human, rat and guinea-pig sera were each dissolved in 0.5 ml of distilled water. The diazo reaction was performed with diazotized *p*-iodoaniline by following the method described by Van Roy *et al.* [13]. Protein interference had been proved to be minimal in this method. Resulting azopigments in the organic phase were analysed by t.l.c. and reversed-phase h.p.l.c. (described below). Azopigments that were not extracted into the organic phase and remained in the aqueous phase were also analysed by the above-described h.p.l.c. gel filtration in 6 M-urea (the absorbance at 530 nm for azopigments instead of 450 nm was recorded).

To assess the possibility of contamination by azopigments derived from non-delta bilirubin, BDG purified from rat bile by phase partition [14] was dissolved in normal rat serum (50 mg/dl) and the mixture was processed in the same manner.

Delta [^{14}C]bilirubin and [^{14}C]bilirubin IX α

In a preliminary study delta [^{14}C]bilirubin and [^{14}C]bilirubin IX α were also diazotized to evaluate the efficacy of the diazo reaction. Cholestasis was induced in Wistar rats by ligation of the bile duct and approx. 50 μCi of 5-amino[^{14}C]laevulinic acid hydrochloride was injected intravenously. After 3 days delta bilirubin labelled with ^{14}C was separated. [^{14}C]Bilirubin IX α was prepared by alkaline hydrolysis of rat bile [15] collected from an indwelling bile-duct cannula after intravenous injection of 50 μCi of 5-amino[^{14}C]laevulinic acid hydro-

chloride into another Wistar rat. [^{14}C]Bilirubin IX α was mixed with rat serum albumin (10 mg for each sample). The diazo reaction was performed as described above, and samples ($n = 3$ for each case) were extracted four times with *n*-butyl acetate. The radioactivity extracted into the organic phase in each wash was measured.

Analysis of azopigments

T.l.c. separation. After the diazo cleavage of delta bilirubin, the upper organic phase was concentrated in a rotary evaporator and developed on a t.l.c. plate. A mixture of chloroform/ethanol/water (65:25:3, by vol.) was used as the developing solvent [13]. Each band on the plate was scraped off and extracted with 1 ml of methanol. The absorbance at 530 nm was measured with a Hitachi model 200-2D spectrophotometer. Azopigments obtained from human or rat bile that was simultaneously processed were used as reference material.

H.p.l.c. analysis [16]. Azopigments were further analysed by h.p.l.c. The organic phase containing azopigments was evaporated *in vacuo* and the resulting azopigments were redissolved in a small amount of methanol. The samples were then applied on an ODS-120A column with a mixture of acetonitrile/50 mM-sodium phosphate buffer (pH 3.0) (13:7, v/v) as the mobile phase. The absorbance at 530 nm was recorded.

To identify the peaks of azopigments on h.p.l.c., azopigments generated from endovinyl and exovinyl BMG were individually applied. These BMG isomers were obtained by the h.p.l.c. separation (as described above) of conjugated bilirubin in rat bile.

RESULTS

Fig. 1 shows serum bilirubins separated by h.p.l.c. gel filtration. Sera were obtained from rats and guinea pigs 3 days after bile-duct ligation. The first peak is of delta

Fig. 1. H.p.l.c. gel filtration of cholestatic sera

H.p.l.c. gel filtration of guinea-pig (left) and rat (right) sera 3 days after bile-duct ligation is shown. A G3000SW column was used, and the eluent was 6 M-urea in 50 mM-sodium phosphate buffer, pH 7.0, at a flow rate of 1.0 ml/min. Delta bilirubin (indicated by *) is co-eluted with albumin and other fractions of bilirubin are eluted later. —, Absorbance at 450 nm ($A_{450} = 0.08$ full scale); -----, absorbance at 280 nm ($A_{280} = 0.64$ full scale).

bilirubin, proved by the co-elution with albumin (detected as an absorbance peak at 280 nm) in 6 M-urea. Other fractions of bilirubin were dissociated from protein and eluted later. As reported previously [9], sera from both jaundiced rats and guinea pigs contained delta bilirubin.

The results of reversed-phase h.p.l.c. analysis of conjugated bilirubin in sera of rats and guinea pigs 3 days after bile-duct ligation are shown in Fig. 2. Conjugated bilirubin in guinea-pig serum was mainly BMG (with a predominance of its endovinyl isomer), whereas that in rat serum consisted of both BDG and BMG, the former usually prevailing. These observations are compatible with previous reports [11,17]. Conjugated bilirubin in jaundiced human sera consisted of both BDG and BMG in various ratios (results not shown).

The results of the diazo reaction of radioisotope-labelled bilirubin are summarized in Table 1. In the case of the diazo reaction of delta [^{14}C]bilirubin, the ratio of

radioactivity extracted into the organic phase through four sequential washes with n-butyl acetate was 35% of the total radioactivity, which was approximately half of the ratio observed when a mixture of [^{14}C]bilirubin IX α and rat serum albumin was similarly processed. The efficiency of the diazo reaction was adequate, as proved by the diazo reaction of [^{14}C]bilirubin IX α . Thus it was indicated that, in the diazo reaction of delta bilirubin, only the dipyrrolic half that was not bound covalently to protein generated azopigments in the organic phase. The azopigments that remained in the aqueous phase were not dissociable from albumin on h.p.l.c. gel filtration in 6 M-urea, suggesting that the other diazotized dipyrrolic half was still bound to albumin.

The t.l.c. separation of azopigments obtained by the diazo reaction of human, rat and guinea-pig delta bilirubin is shown in Fig. 3. All azopigments were proved to be derived exclusively from delta bilirubin, since the

Fig. 2. Fractions of conjugated bilirubin in cholestatic sera

Reversed-phased h.p.l.c. analysis of conjugated bilirubin in jaundiced sera of guinea pig (a) and rat (b). The solid phase was ODS-120A, and the mobile phase was 50 mM-citrate buffer (pH 5.0)/methanol/acetonitrile (8:9:3, by vol.), at a flow rate of 0.6 ml/min. Absorbance at 450 nm ($A_{450} = 0.04$ full scale) is shown.

Table 1. Radioactivity in the organic phase after the diazo reaction

After the diazo reaction of delta [^{14}C]bilirubin and [^{14}C]bilirubin IX α , the azopigments were extracted with n-butyl acetate four times. The radioactivity in the organic phase was individually counted. The radioactivity in the organic phase was then calculated as a percentage of the radioactivity in the original mixture. Means $\pm$ s.d. for three experiments are shown.

Sample	Sequential wash ...	Radioactivity in organic phase (% of total)				Total
		1	2	3	4	
Delta [^{14}C]bilirubin		22.9 $\pm$ 3.2	7.6 $\pm$ 2.0	2.7 $\pm$ 0.4	2.0 $\pm$ 1.0	35.8 $\pm$ 0.8
[^{14}C]Bilirubin IX α		47.7 $\pm$ 3.9	14.0 $\pm$ 1.0	13.8 $\pm$ 1.9	3.1 $\pm$ 1.5	78.6 $\pm$ 1.7

Fig. 3. T.l.c. analysis of azopigments

T.l.c. analysis of azopigments derived from (a) guinea-pig delta bilirubin, (b) rat delta bilirubin, (c) and (d) human delta bilirubin and (e) human bile is shown. The developing solvent was chloroform/ethanol/water (65:25:3, by vol.). Two bands on (a) indicated by * were of yellow pigment of unknown origin.

mixture of BDG and normal rat serum yielded no azopigments after being washed with caffeine/benzoate reagent. The azopigment derived from guinea-pig delta bilirubin yielded only one band, which was identified as unconjugated azodipyrrole by comparison with reference material. Human and rat delta bilirubin generated two bands of azopigment with different polarities. The more polar one, which did not appear in the diazo reaction of guinea-pig delta bilirubin, was identified as azodipyrrole monoglucuronide. This conjugated azopigment accounted for 20–50% of total azopigments spectrophotometrically.

As shown in Fig. 4, azopigments obtained from exovinyl BMG were separated by h.p.l.c. into two peaks, representing exovinyl azodipyrrole monoglucuronide and unconjugated endovinyl azodipyrrole respectively. Azopigments from endovinyl BMG were separated into two peaks of endovinyl azodipyrrole monoglucuronide and unconjugated exovinyl azodipyrrole, and these four peaks were distinguishable when azopigments from both isomers of BMG were eluted together.

The result of h.p.l.c. analysis of azopigments derived from rat delta bilirubin is shown in Fig. 5(b). Two major peaks with lesser polarity were identified as both endovinyl and exovinyl isomers of unconjugated azodipyrrole. These peaks usually had about the same amplitude. Two other major peaks with greater polarity were identified as the isomers of azodipyrrole monoglucuronide. They also had nearly the same amplitude. The analysis of azopigments derived from guinea-pig delta bilirubin is shown in Fig. 5(a), with major peaks of unconjugated exovinyl and endovinyl azodipyrroles and trace amounts of conjugated ones. The peak of the unconjugated exovinyl azodipyrrole was considerably greater than that of the endovinyl one when guinea-pig delta bilirubin was diazotized.

DISCUSSION

Lauff and his colleagues analysed azopigments obtained from human delta bilirubin [4,5] by diazo reaction

Fig. 4. H.p.l.c. analysis of azopigments

Identification of four bands of azopigments on reversed-phased h.p.l.c. is shown. (a) Azopigments derived from exovinyl BMG; (b) azopigments derived from endovinyl BMG; (c) azopigments derived from both exovinyl BMG and endovinyl BMG. The solid phase was ODS-120A, and the mobile phase was acetonitrile/50 mM-sodium phosphate buffer (pH 3.0) (13:7, v/v), at a flow rate of 0.85 ml/min. Absorbance at 530 nm ($A_{530} = 0.01$ full scale) is shown. Abbreviations: c-, glucuronide-conjugated; u-, unconjugated; exo, exovinyl azodipyrrole; endo, endovinyl azodipyrrole.

with diazotized dichloroaniline [18]. They reported that half of the resulting azopigments moved into the organic phase and the rest remained in the aqueous phase still bound to protein. The extracted azopigment in the organic phase, which originated from the dipyrrolic half not bound covalent to protein, was reported to be mainly unconjugated. Gross *et al.* [19] also analysed azopigments obtained from human delta bilirubin by diazo reaction

Fig. 5. Azopigments obtained from delta bilirubin

H.p.l.c. analysis of azopigments obtained from delta bilirubin of guinea pig (a) and rat (b) is shown. Absorbance at 530 nm ($A_{530} = 0.01$ full scale) is shown. Conditions and abbreviations are the same as in Fig. 4.

with diazotized ethyl anthranilate and reported similar results.

Although the mechanism of formation of delta bilirubin is not clarified, McDonagh and co-workers suggested that covalent binding of bilirubin to albumin occurs through acyl migration of bilirubin from a bilirubin glucuronic acid ester to a nucleophilic site on albumin [9]. This would imply that delta bilirubin is derived mainly from monoconjugated bilirubin, since the dipyrrolic half of delta bilirubin that is not bound covalently to protein was reported to be essentially unconjugated.

In the present work we used diazotized *p*-iodoaniline to perform diazo reaction on delta bilirubin obtained from guinea-pig, rat and human jaundiced sera. The efficiency of the diazo reaction was adequate, as proved by the diazo reaction of [^{14}C]bilirubin IX α . In the diazo reaction of delta bilirubin, the diazotized dipyrrolic half not bound covalently to protein was extracted in the organic phase, and the other dipyrrolic half remained in the aqueous phase with albumin. Further analyses were concentrated on the azopigments in the organic phase.

In our study, guinea-pig delta bilirubin generated only unconjugated azodipyrrole, whereas human and rat delta bilirubin generated both azodipyrrole mono-glucuronide and unconjugated one. This result, inconsistent with previous reports, provides us with two suggestions concerning the structure and the production mechanism of delta bilirubin.

First, since delta bilirubin is generated exclusively from conjugated bilirubin and BMG predominated in jaundiced guinea pig serum, it is reasonable to conclude that the delta bilirubin of guinea pigs is derived mainly from BMG. Furthermore, our finding that the diazo reaction of guinea-pig delta bilirubin generated only unconjugated azodipyrrole indicates that covalent binding of bilirubin to protein occurs on the conjugated

dipyrrolic half of parent BMG, supporting the suggestion by McDonagh *et al.* [9].

Secondly, conjugated azodipyrrole obtained from human and rat delta bilirubin indicates the existence of 'conjugated' delta bilirubin, preserving glucuronic acid on the dipyrrolic half not bound covalently to protein. This observation suggests that delta bilirubin originates not only from BMG but also from BDG. The ratio of 'conjugated' delta bilirubin to 'unconjugated' delta bilirubin was usually smaller than the BDG/BMG ratio in the sera. There is a possibility of deconjugation of 'conjugated' delta bilirubin *in vivo* or *in vitro*, and whether BMG and BDG have different affinities for delta bilirubin formation remains to be investigated.

Reversed-phase h.p.l.c. analyses of azopigments derived from delta bilirubin further revealed that delta bilirubin originates from both endovinyl and exovinyl isomers of BMG, as the endovinyl isomer of unconjugated azodipyrrole in the organic phase indicates that covalent binding with protein occurred on the exovinyl dipyrrolic half of original delta bilirubin and vice versa. The predominance of exovinyl azodipyrrole in the case of guinea-pig delta bilirubin is explained by the fact that endovinyl BMG prevails in the jaundiced guinea pig [11]. Our study concerning human and rat delta bilirubin also indicated that BDG binds to protein on either endovinyl or exovinyl dipyrrolic half with equal probability in these animals. So there are at least four forms of delta bilirubin. Conjugated bilirubin other than glucuronide may also generate delta bilirubin.

In conclusion, the analyses of azopigments obtained from delta bilirubin in human, rat and guinea-pig sera indicated that delta bilirubin originates from any form of bilirubin glucuronide, i.e. endovinyl and exovinyl isomers of BMG and BDG. Delta bilirubin generated from BMG did not possess glucuronic acid, whereas that generated from BDG existed as 'conjugated' delta bilirubin,

preserving the glucuronic acid on the dipyrrolic half not bound covalently to protein.

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