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Note

Reaction of bilirubin glucuronides with serum albumin

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Increases in serum levels of bilirubin covalently bonded to albumin (called δ-bilirubin or bilirubin–albumin) have recently been found to be associated with a number of clinical circumstances in which normal secretion of bilirubin glucuronide into the bile is impaired [1]. These include bile duct obstruction, hepatitis, cirrhosis, infiltrative disease, sepsis and the Dubin–Johnson syndrome. Attention has been called by these same authors [1] that the levels of δ-bilirubin are not raised in clinical conditions such as neonatal jaundice in which clinically visible jaundice is primarily due to non-conjugated bilirubin in the circulation. Furthermore, since this investigation began, Gautam et al. [2] have reported the irreversible binding of conjugated bilirubin to albumin in cholestatic rats. McDonagh et al. [3] also reported evidence of formation of bilirubin–albumin.

Our interest in the covalent bonding of bilirubin to albumin was attracted because of on-going studies of chemical reactivities of glucuronides formed as drug metabolites. We propose a non-enzymatic basis for the production of the bilirubin–albumin adduct which is consistent with and explains the clinical pattern. We have shown that metabolites of a number of drugs in which carboxylic acid groups are conjugated as glucuronides undergo spontaneous transacylation to form covalent bonds beteen the drug and chemical nucleophiles, including bovine serum albumin [4–7]. In this context it seemed likely to us that bilirubin

monoglucuronide and diglucuronide, also being conjugated through carboxylic acid groups, could react chemically with nucleophilic centers in human serum albumin to produce covalently bonded bilirubin–albumin. The present report provides evidence confirming this hypothesis from studies with isolated bilirubin conjugates and isolated albumins.

EXPERIMENTAL

Preparation of sulfhydryl-blocked serum albumin

Free sulfhydryl groups on cysteine residues of Pentex crystalline human serum albumin (Miles Scientific, Naperville, IL, U.S.A.) were blocked by reaction with iodoacetamide (Sigma, St. Louis, MO, U.S.A.). The albumin–acetamide product was dialyzed against 0.9% saline solution in order to remove iodide and unreacted iodoacetamide. The solution was made 0.13 mM in albumin–acetamide and used for incubation with bilirubin conjugates as described below.

Isolation and characterization of the conjugates of bilirubin

The bilirubin conjugates (mono- and diglucuronides) were isolated from bile of pentobarbital-anesthetized rats, whose common bile duct had been cannulated (PE-10 tubing). The rats were infused with 5 mg of bilirubin (Sigma) dissolved in an isotonic aqueous solution of sodium carbonate and sodium chloride as previously described [8]. The bile was directly applied to silica gel plates (Analtech, Newark, DE, U.S.A.; 20×20 cm, 250 μm thickness) and developed to 19 cm in ethyl acetate and air dried. The plates were subsequently developed to 18 cm in chloroform–methanol–water–glacial acetic acid (60:34:6:0.2). The bands of regioisomeric mono- and diglucuronides were scraped from the plates and eluted from the silica with 0.13 mM human albumin or 0.13 mM albumin–acetamide solution in 0.9% saline. Two successive elutions of 1.0 ml each were used, which were combined and then centrifuged at 4000 g at 4°C to remove remaining silica particles. This purification and elution protocol brought albumin into contact with 1-O-acyl glucuronides from fresh bile immediately after separation by thin-layer chromatography (TLC).

All bile collections, chromatographic separations and elutions were performed in subdued light (safe-lamps). Bilirubin glucuronide and albumin concentrations of the eluates were determined according to previously published procedures [8]. Bilirubin mono- and diglucuronides were identified by comparison with authentic standards on TLC. They were further characterized by reaction with diazotized sulfanilic acid [9].

High-performance liquid chromatographic (HPLC) analyses after incubation and storage indicated that the bilirubin diglucuronide fraction contained less than 5% monoglucuronides, and bilirubin monoglucuronide samples contained less than 10% diglucuronide. Chromatograms of bilirubin monoglucuronides also showed two nearly resolved symmetrical monoglucuronide peaks, which presumably reflected conjugation with either one of the two propionic acid groups of the asymmetric bilirubin molecule. The diglucuronide peaks, analyzed after incuba-

tion and storage, had two small shoulders suggesting that some intramolecular isomerization had taken place.

Incubation of the bilirubin conjugates and albumin

One aliquot (1 ml) of each TLC eluate in 0.13 mM albumin or albumin–acetamide and 0.9% saline was immediately frozen at $-20°C$ (no incubation at 37°C), and the remainder (1 ml) was incubated in a shaking water bath for 75 or 120 min and then frozen at $-20°C$. The frozen samples were lyophilized overnight and maintained at $-20°C$ until further analysis. Control samples included 0.13 mM serum albumin, 0.13 mM albumin–acetamide or 0.13 mM albumin with non-conjugated bilirubin, 0.11 mM, treated as above. The incubation mixtures of glucuronides and controls were analyzed by HPLC.

High-performance liquid chromatography

The incubation mixtures were analyzed on an Altex 322 liquid chromatograph (Beckman Instruments, Berkeley, CA, U.S.A.) equipped with a Brownlee Labs. (Santa Clara, CA, U.S.A.) Aquapore RP-300 analytical column (25 cm × 4.6 mm) and a Brownlee Labs. Aquapore RP-300, MPLC guard column maintained at 40°C. The solvent system of Lauff et al. [10] was used. Solvents were HPLC grade and were filtered and degassed under vacuum before use. Column effluents were monitored at 450 nm using a Schoeffel (Westwood, NJ, U.S.A.) Model SF770 variable-wavelength detector. Three injections were made per analysis, each containing 225 μg of human serum albumin. Estimates of the concentrations of bilirubin were based on comparisons of their peak heights with those of standard concentrations of bilirubin monoglucuronides in the same chromatography system.

RESULTS

The chromatographic system used here to analyze bilirubin–albumin is similar to that which was used to demonstrate the existence of covalently bonded bilirubin–albumin [2,3,11] and was used subsequently to analyze the association of this conjugate with various disease states [1]. In this system, bilirubin–albumin coelutes with albumin. Only the former is reported to absorb strongly in the region 436–450 nm. Consequently, the identification of bilirubin–albumin is based on increases in absorbance relative to that of albumin in control reactions. The absorbance recorded for control albumin is high relative to the cleavages. The tailing absorbance at 450 nm of a 13 mM solution of the same albumin measured using a scanning spectrophotometer, 1-cm cell, saline reference, was found to have an extinction coefficient of 0.03. The absorbance of control albumin was not altered by the presence of unconjugated bilirubin, nor by derivatization of the free sulfhydryl group (Table I).

Incubation of serum albumin with unconjugated bilirubin produced no increase in the absorbance of albumin at 450 nm. Reaction of albumin with iodoacetamide also produced no increase in its absorbance at 450 nm. Quantitative assessments of the bilirubin–albumin peaks are presented in Table I. The data demonstrate

TABLE I

ABSORBANCE AT 450 nm FOR ALBUMIN REACTED WITH BILIRUBIN GLUCURONIDES

Bilirubin and bilirubin glucuronides were incubated with 0.13 mM human serum albumin, 75 min at 37°C except as noted.

Albumin reaction mixture	Concentration (mM)	Absorbance at 450 nm (a.u.f.s. ± R.S.D.)
Bilirubin diglucuronide	0.36★	0.0538 ± 0.0011
	0.091	0.0440 ± 0.0008
	0.030	0.0365 ± 0.0004
Bilirubin monoglucuronide	0.21	0.0372 ± 0.0007
	0.11	0.0350 ± 0.0005
	0.032★★	0.0331 ± 0.0008
	0.032	0.0346 ± 0.0001
	0.033★★,★★★	0.0326 ± 0.0005
	0.033★★★	0.0338 ± 0.0001
Controls		
Bilirubin	0.11	0.0310 ± 0.0002
Serum albumin	0.13	0.0318 ± 0.0008
Albumin-acetamide	0.13	0.0313 ± 0.0006

★Incubated for 120 min.
★★No incubation at 37°C.
★★★Incubated with 0.13 mM albumin-acetamide.

that covalently bonded chromophoric material was eluted with albumin which had been incubated with bilirubin glucuronides. The formation of bilirubin–albumin increased as the concentration of bilirubin glucuronide in the incubation increased. This covalently bonded bilirubin–albumin was not dialyzable or reversibly bound material [11]. Bilirubin–albumin retained its reactivity with diazotized sulfanilic acid, characteristic of the methylene bridge in bilirubin, as determined by HPLC with detection at 450 and 540 nm.

Reaction mixtures containing albumin and bilirubin monoglucuronide were analyzed without incubation as well as with incubation at 37°C. Both groups of samples were found to contain covalently bonded bilirubin–albumin. Incubation at 37°C for 75 min increased the bilirubin–albumin content 40–50% over non-incubated samples, which already contained considerable amounts of products. (See examples in Table I.) Thus reaction of serum albumin with bilirubin glucuronides appeared to occur rapidly upon mixing, with further reaction at a much slower rate. This sequence was also found in the acylation of albumin by flufenamic glucuronide [5] and is proposed to reflect initial high affinity binding at specific site(s) on albumin.

The role of free cysteine sulfhydryl groups, one obvious candidate for the site of reaction of albumin with bilirubin glucuronides, was investigated using albumin whose free sulfhydryl group had been modified by reaction with iodoacetamide. Results are presented in Table I for bilirubin monoglucuronide. Both types of albumin reacted immediately with bilirubin glucuronides even without incu-
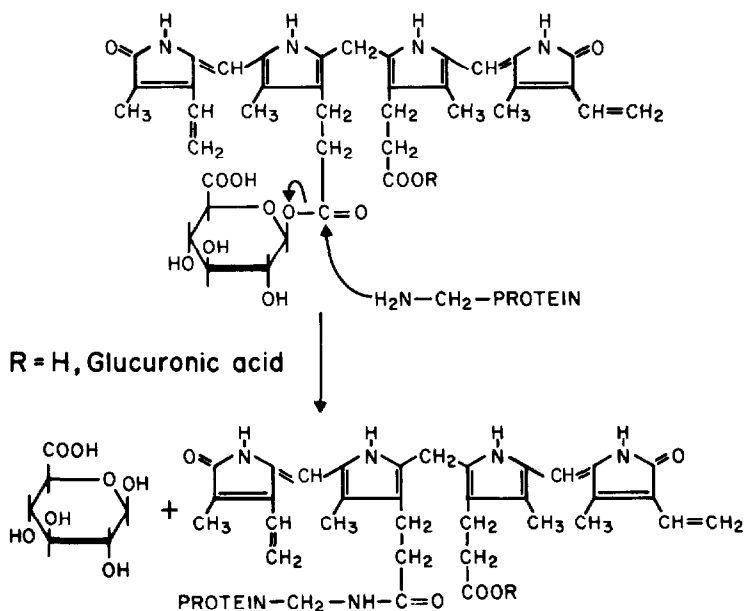
Fig. 1. Proposed scheme for the transacylation of bilirubin glucuronide by serum albumin.

bation at 37 °C. Sulfhydryl-blocked albumin appeared to react to a lesser extent than unmodified albumin. However, the formation of covalent bonds with bilirubin was clearly not limited to cysteine residues. This has also been shown for the reaction of albumin with flufenamic 1-O-acyl glucuronide [5].

The concentrations of bilirubin—albumin whose formation is summarized in Table I ranged from approximately 5 μM for the reaction of 0.033 mM monoglucuronide with albumin—acetamide with no incubation to 90 μM for the reaction of 0.36 mM diglucuronide with albumin with 120 min incubation.

DISCUSSION

Our results demonstrate the spontaneous reaction in vitro of isolated bilirubin mono- and diglucuronides with human serum albumin to produce covalently bound bilirubin—albumin. The reaction presumably proceeds with the displacement by one or more nucleophilic functional groups on the albumin molecule of glucuronic acid from its carboxylic acyl linkage with bilirubin (Fig. 1). This mechanism is consistent with the observation that bilirubin—albumin, like bilirubin, reacts with diazotized sulfanilic acid. Furthermore, bilirubin diglucuronide, which contains two electrophilic acyl bonds, was found to be more reactive towards albumin than the monoglucuronide, which is also consistent with a transacylation mechanism for the formation of bilirubin—albumin. The mechanism can explain the reactivities which we have determined for the 1-O-acyl glucuronides of clofibric acid [4], benoxaprofen, flufenamic acid, indomethacin [5] and other glucuronides [6,7]. This agrees with previous observations of facile ammonolysis, methanolysis [12] and internal isomerization/transacylation [13,14] of bilirubin glucuronides.

The exact structures of the transacylation products remain to be worked out, including details of bilirubin regioisomerism and the identities and locations of alkylated amino acid units in albumin. The rates of product formation and subsequent stability require quantitative assessment before the chemical basis for this interesting physiological transformation is completely understood. Nevertheless, in view of the larger picture evolving of acyl-linked glucuronide reactivities, it is highly probable that the reactivity demonstrated here in vitro also occurs in vivo. Formation of covalent bilirubin–albumin might be expected to be significant only when the concentration of bilirubin glucuronides in the serum is abnormally increased. This hypothesis is supported by the clinical observation of bilirubin–albumin exclusively in cases of conjugated hyperbilirubinemia [1].

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