The Effect of Exogenous Conjugated Bilirubin on the Urinary Excretion of Unconjugated ^{14}C-Bilirubin by the Gunn Rat[1]

In man, and in several animal species, unconjugated bilirubin (UB) is rapidly conjugated as the diglucuronide (CB) or pigment II[2]. A monoglucuronide or pigment I has also been described[3,4] but it is now considered as an equimolecular complex of UB and CB[5]. Intravenous injection of UB increases the concentration of pigment I[3,5] or of UB[6] in the bile. It has not yet been clarified whether hydrolisis of CB occurred or the complex was formed. As far as we know, no evidence has been presented on the in vivo synthesis of such a complex. The data reported in this paper support the existence of the complex.

We have used the Gunn rat as experimental model, due to its congenital defect of glucuronoconjugation[7] and despite the fact some CB has been observed in bile[8]. The urinary excretion of labelled UB in Gunn rats with bile duct ligation and injected with unlabelled CB was studied. Since CB is selectively excreted in urine, the increase of labelled UB in urine after the injection of unlabelled CB, has been taken as evidence of complex formation (Figure 1).

Methods. Labelled UB was obtained in 2 ways: (a) by injecting ^{14}C-ALA (δ-aminolevulinic acid-4-^{14}C hydrochloride, CEA, France) s.c. in 6 Gunn rats as a single dose (5 microcuries per animal) and, (b) by i.v. injection of ^{14}C-bilirubin in 27 Gunn rats (100–145 µg, specific activity 2000–3200 DPM/µg) crystallized from bile[9] of normal rats injected with ^{14}C-ALA. Some of the rats also received different quantities of CB (100–2200 µg) in the form of bile from normal rats injected with UB i.v.

The animals were allowed access to 5% glucose solution ad libitum and placed in restraining cages. Urine and blood were collected during 6–24 h and at the end of the experiment respectively. Urine and serum samples were stored at −15 °C until analyzed. Determinations of urinary bilirubin[10] and of serum bilirubin[11] were carried out in a DU Beckman spectrophotometer. Crystallizations of bilirubin and measurements of radioactivity in a Packard Tri Carb Liquid Scintillation Spectrometer were performed as described[7,9]. Paper chromatography of diazotized samples of urine were carried out[12]; the samples were concentrated to a small volume[13] and the azopigments purified as reported[14]. Reverse phase partition chromatography of pooled urine samples was also performed[15] and the pigments eluted from the column, diazotized and then chromatographed on paper. Azopigments spots were finally eluted from paper with 1.5% HCl (v/v), extracted in butanol[13], the specific activity measured and compared to that of serum azopigments.

Results. In the Gunn rats, injected simultaneously with ^{14}C-ALA and unlabelled CB, a significant increase of radioactive urinary bilirubin ($P < 0.01$) and of total urinary bilirubin were observed (Figure 2 and Table).

In the experiments where ^{14}C-bilirubin and unlabelled CB were administered simultaneously, urinary radioactivity and total bilirubin were both also increased. Animals receiving ^{14}C-bilirubin and CB excreted 3.3% (S.D. ± 0.8) of the dose after 6 h and 7.1% (S.D. ± 0.8) after 21 h. These values are more than twice those observed in control rats, the differences being statistically significant ($P < 0.001$). Crystallization of bilirubin from urine collected in paired experiments, revealed that ^{14}C-bilirubin excretion was increased 2–5 times in rats injected with CB. When CB was given in the form of bile samples

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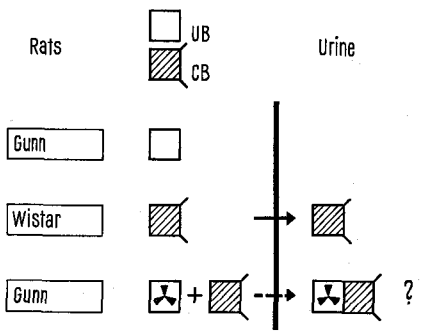

Fig. 1. Diagram showing the theoretical explanation for which evidence is presented regarding the effect of CB on labelled UB excretion in the urine of Gunn ligated rats.

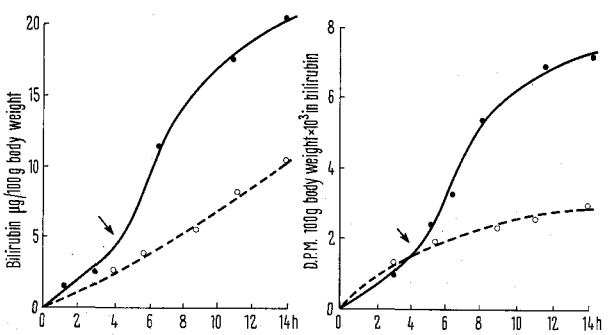

Fig. 2. Urinary excretion of total bilirubin and radioactivity in bilirubin, in Gunn rats with bile duct ligation and injection of ^{14}C-ALA. ●, Gunn rat injected with unlabelled CB; ○, control Gunn rat; the arrow indicates the time of injection.

Effect of CB on the urinary excretion of bilirubin by Gunn rats with bile duct ligation and injection of ^{14}C-ALA

Rat	CB injected (μg/100 g)	Interval of collection (h)	Urinary bilirubin (μg/100 g)	Radioactivity in bilirubin (DPM/100 g)
1	–	10	8	1210
2	–	9	4	2530
3	–	9	11	710
4	340	9.5	17	5080
5	530	9	38	5250
6	350	9	42	5480

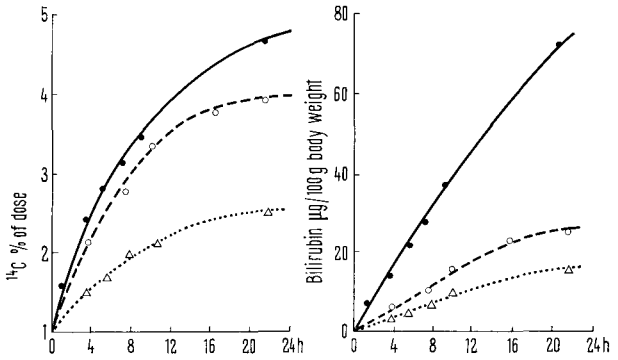

Fig. 3. Urinary excretion of total bilirubin and radioactivity as a percent of dose, in Gunn rats with bile duct ligation and injection of bile samples containing constant amounts of bile salts and different concentrations of CB: △, injection of 30 μg; ○, injection of 130 μg; ●, injection of 500 μg.

containing constant amounts of bile salts[16] and different concentrations of CB, urinary radioactivity and bilirubin were found to be a function of the dose of CB (Figure 3).

Chromatograms of diazotized urine samples of rats that received CB showed azopigments A and B; scanning of the paper strips revealed a major peak of radioactivity over azopigment A (Figure 4). After purification of azopigments, 100% of radioactivity was associated to azo A spot. Reverse phase partition chromatography of pooled urine samples from rats injected with both ^{14}C-bilirubin and unlabelled CB demonstrated the presence of bilirubin, pigment I and pigment II. When eluted, diazotized and chromatographed on paper, they gave respectively azopigment A; A + B, and B plus an orange spot probably corresponding to water-soluble catabolites (Figure 5). Ratio of specific activity between azo A and B spots from bilirubin and pigment II respectively, and between azo A and B spots from pigment I, was 3:1 in both cases. Urine samples from control animals also showed azopigments A and B on paper chromatography, but the specific activity of azopigment A was very much lower than in rats injected with CB, the differences being equal to that of ^{14}C-bilirubin excretion. Specific activity of azo A spots separated by paper chromatography of serum samples, and radioactivity in total serum bilirubin were similarly lower in the control group.

Finally, in a couple of paired experiments with rats receiving or not CB, chemical partition of serum direct-reacting pigments[17], revealed more than twice of pigment I in the treated group.

These experiments demonstrate that CB is able to increase urinary excretion of radioactive UB in the Gunn rat. This phenomenon can be explained through the previous formation of a complex between both pigments.

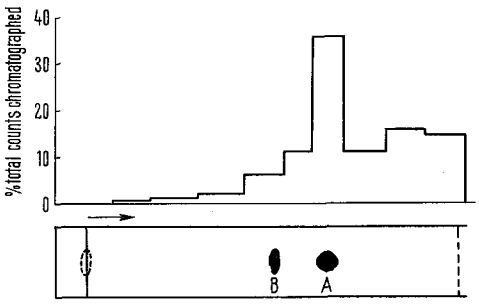

Fig. 4. Chromatography of diazotized urine collected from a Gunn rat with bile duct ligation that received ^{14}C-bilirubin and CB.

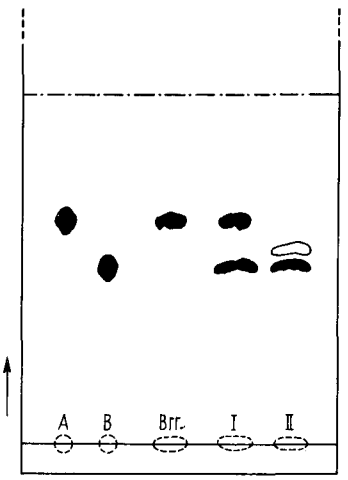

Fig. 5. Paper chromatography of diazotized pigments after separation by reverse phase partition chromatography. A and B standard azopigments; Brr, bilirubin at the top of the column; I, pigment with R 0.3; II, pigment collected with the solvent front.

Resumen. La inyección de bilirrubina conjugada a ratas Gunn con ligadura coledociana, produce un aumento de la excreción urinaria de bilirrubina C^{14} exógena o de la endógena sintetizada a partir de la inyección de ALA-C^{14} en dichos animales. Dicho aumento fue probado por la cristalización de la bilirrubina radioactiva de muestras de orina y por la radioactividad presente en el azopigmento A separado por cromatografía sobre papel. El aumento de pigmento I, demostrado en el suero por partición química y en la orina por columna cromatográfica de fase inversa, permiten suponer la formación de un complejo de bilirrubina no conjugada radioactiva y de bilirrubina conjugada, como mecanismo del pasaje mencionado.

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