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FORMATION OF BILIRUBIN MONOGLUCURONIDE AND DIGLUCURONIDE IN ISOLATED RAT HEPATOCYTES

EFFECT OF SPIRONOLACTONE

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Abstract—The formation of bilirubin monoglucuronide (BMG) and diglucuronide (BDG) was studied in isolated rat hepatocytes with appropriate viability. Isolated cells were obtained from normal rats and from rats pretreated with spironolactone (SP). A fixed number of cells (4.8×10^6) was incubated in a medium containing uridine diphosphoglucuronic acid (UDPGA, 3.4 mM) and bilirubin (11.3 μM, 29 μM, 50 μM and 81 μM) for different time intervals (from 0 to 25 min). The pellet of cells and the supernatant fraction were subjected to alkaline methanolysis, and the proportions of BMG and BDG were estimated by thin-layer chromatography. No conjugates were detected at time O or in the absence of UDPGA in the incubation system. BMG and BDG were detected after 2 min of incubation and then they increased up to 15 min of incubation. Both conjugates were mostly found in the supernatant fraction, and a predominance of BMG was apparent. Normal cells also synthesized increasing amounts of BMG and BDG with the increase of bilirubin substrate concentration up to 50 μM. When hepatocytes from SP-treated rats were used, a more rapid rate of glucuronidation, that was mainly produced at the expense of BMG found in the supernatant fraction, was clear. The results probably indicate that enzymic conversion of BMG to BDG may be rate limiting in isolated hepatocytes although other possible mechanisms were not excluded.

It is well established in man and other species that bilirubin is excreted in bile mainly in the form of diconjugates of glucuronic acid [1–3]. However, by using liver broken-cell preparations, it was observed that synthesis of bilirubin conjugates with glucuronic acid was produced largely at the expense of bilirubin monoglucuronide (BMG) [4–6], and that preferential formation of BMG *in vitro* was related to the high bilirubin substrate concentration used [7]. It has been postulated that conversion of bilirubin to BMG is catalyzed by microsomal UDP-glucuronyl transferase (EC 2.4.1.17) [8], but conflicting results were reported concerning the mechanism of bilirubin diglucuronide (BDG) synthesis [9–12]. In previous investigations, we observed that spironolactone (SP) increased bilirubin glucuronidation in rat liver and small intestinal mucosa homogenates, and that SP pretreatment resulted in a more rapid rate of BDG excretion into the bile of living rats and isolated perfused rat livers [13, 14]. Therefore, the purpose of this study was 2-fold: (1) to determine the formation of BMG and BDG in isolated rat hepatocytes, a model resembling the situation *in vivo* more closely than other preparations and (2) to examine the effect of SP pretreatment on the formation of both conjugates by isolated cells.

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MATERIALS AND METHODS

Chemicals. All the chemicals used were of reagent grade quality. Collagenase type IV, Trypan blue, bilirubin, SP, bovine albumin (fraction V), uridine diphosphoglucuronic acid (UDPGA), and Triton X-100 were purchased from the Sigma Chemical Co. (U.S.A.). Silica gel G (Kieselgehl 60 G) was from Merck (West Germany), and monotest LDH opt., test UV was from Boehringer GmbH, Mannheim (West Germany).

Animal and drug treatment. Male Wistar rats weighing 220–260 g were used. SP was injected i.p. as a daily dose of 240 μmoles/kg body wt dissolved in propylene glycol for 3 consecutive days prior to the experiment [13]. Control rats were injected with propylene glycol. The animals were allowed free access to water and saline solution during treatment and were maintained *ad lib*. on a standard laboratory pellet diet.

Preparation of hepatocytes. Hepatocytes were isolated according to the procedure of Berry and Friend [15] and Seglen [16], with some modifications. Rats were anesthetized by an i.p. injection of sodium pentobarbital (50 mg/kg body wt). After a midline abdominal incision, the liver was exposed, the animal was heparinized, and the vena porta was cannulated with a PC-100 catheter (P. L. Rivero & Cia, Argentina). The liver was perfused *in situ* with Ca^{2+}- and Mg^{2+}-free Hanks' buffer for 6–8 min in a thermostatically controlled chamber at 37°, and at a rate of

18–23 ml/min. The perfusion medium was continuously bubbled with a gas mixture (95% O_2–5% CO_2). During the perfusion, hepatic lobes were gently manipulated by hand to facilitate complete washout of blood and extracellular calcium. Then the perfusion medium was replaced by 250 ml of the same buffer but containing 0.05% (w/v) collagenase, and the perfusion was continued at a rate of 10–12 ml/min. The perfusion medium was constantly gassed with carbogen and did not recycle. At the end of the perfusion, the liver was transferred to a plastic reservoir containing Hanks' buffer with calcium and 0.05% (w/v) collagenase, pH 7.4, and gently disrupted with a glass rod. The formed suspension was incubated in a Dubnoff metabolic shaker (37°, 10 min, 50 strokes/min, in a carbogen atmosphere). Afterwards, the suspension was filtered through a nylon layer and the filtrate was centrifuged (50 *g*, 90 sec). The pellet was washed with Ca^{2+}- and Mg^{2+}-free Hanks' buffer to obtain a suspension free of calcium to prevent the formation of precipitates by calcium and bilirubin during incubation [17]. Filtration, centrifugation, and pellet washing were repeated three times. Cell viability after isolation was estimated by Trypan blue exclusion and lactate dehydrogenase (LDH) release. The ability of the cells to exclude the dye was determined microscopically counting the number of stained and nonstained cells in an improved Neubauer chamber. Trypan blue staining showed that more than 84% of the cells did not take up the dye. LDH released by cells averaged 15% of total enzyme measured after the lysis of cells with 0.1% Triton X-100 [18]. The cell suspension was stored on ice until the assay was commenced within 30 min of its preparation.

Incubations. The incubation medium consisted of cell suspension, $MgCl_2$, bilirubin–albumin substrate, and UDPGA in a final volume of 1.5 ml. Incubation mixtures were prepared at 0° in glass-stoppered centrifuge tubes as follows. (a) One milliliter of hepatocyte suspension with a concentration of 4.8×10^6 cells/ml was used in all experiments. (b) The concentration of $MgCl_2$ in the incubation medium was 4.17 mM. (c) Incubation mixtures contained appropriate amounts of bilirubin (17, 43.5, 75 and 121.5 nmoles) to obtain four different bilirubin concentrations in the incubation system (11.3 μM, 29 μM, 50 μM and 81 μM). Cell viability was shown not to be affected significantly by bilirubin within the concentration range used up to 25 min of incubation. Bilirubin was dissolved previously in 0.05 NaOH in the dark, and then it was diluted in a bovine albumin solution [5]. The ratio of bilirubin to albumin was constantly 0.63. (d) The optimal amount of UDPGA in the incubation system was investigated previously. Figure 1 shows total bilirubin conjugates (BMG + BDG) present in cells and medium after 15 min of incubation of cells in a medium containing 50 μM bilirubin. From such experiments the optimal concentration of UDPGA was found to be 3.4 mM, which was used in all experiments. The bilirubin–albumin substrate and the UDPGA solution were prepared immediately before each experiment. The reaction was begun by adding cell suspension and transferring the incubation tubes to a Dubnoff metabolic shaker at 37°. A control tube was taken through the procedure, and it contained the above-mentioned components except that water was substituted for UDPGA. The control was incubated at the same time as the experimental tubes and was treated thereafter in identical fashion.

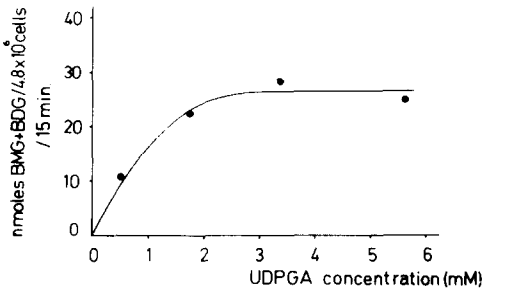

Fig. 1. Effect of varying the concentration of UDPGA in the incubation mixture on bilirubin glucuronidation by normal hepatocytes. Each point is the mean of two separate experiments. The ordinate represents the sum of BMG and BDG determined in cells and supernatant fraction after 15 min of incubation. The bilirubin substrate concentration in the incubation mixture was 50 μM.

Determination of BMG and BDG. All the steps were constantly carried out in a dark room. Illumination with red light was used only when necessary. At the end of the incubation, control and test tubes were centrifuged (50 *g*, 90 sec). The pellet of cells (after washing twice with the Ca^{2+}- and Mg^{2+}-free Hanks' buffer) and 0.4 ml of the supernatant fraction were subjected to alkaline methanolysis [3] by adding a mixture containing methanol (2 ml), sodium ascorbate (20 mg) and a trace of EDTA, and then 2 ml of 2% KOH in methanol (w/v). After mixing, the tubes were allowed to remain at 20–25°, for 1 min, and then 2 ml chloroform (redistilled) and 4 ml glycine–HCl buffer, pH 2.7 (prepared by adding solid glycine to 0.4 M HCl, at 25°), were added. The mixture was centrifuged (800 *g*, 5 min), and the chloroform extract was pipetted off into cuvettes with a 1-cm light path. The absorbance at 450 nm was determined spectrophotometrically (Varian 634 S, Australia) against a blank of chloroform. Absorbance value were converted into micrograms of total bilirubin extracted, by use of the molar extinction coefficient for bilirubin [3]. When known amounts of bilirubin were added to the incubation mixture (from 40 to 122 nmoles), nearly 90% of the pigment was recovered in the respective chloroform extracts of cells and supernatant fractions subjected to the alkaline methanolysis after 15 min of incubation. Recovery of added bilirubin in chloroform extracts after alkaline methanolysis decreased to 70% when a lower amount of pigment (17 nmoles) was incorporated in the incubation mixture. For determination of BMG and BDG, chloroform extracts were concentrated by evaporation under N_2 at 30° and immediately applied to thin-layer chromatography plates (5 × 20 cm) precoated with silica gel. The plates were developed with chloroform–methanol–acetic acid (97:2:1, by vol.) for 17–18 cm. The bilirubin, BMG and BDG bands [3] were immediately scraped from the plates, and the pigments were eluted with 1 ml of chloroform–methanol (1:1, v/v).

Absorbance values of the eluates were determined spectrophotometrically at 450 nm. Relative values were referred to total bilirubin values determined in chloroform extracts and thus expressed as nmoles of bilirubin, BMG and BDG per 4.8×10^6 cells present in the pellet of cells, the supernatant fraction, or the sum of the two, and per each incubation time (from 0 to 25 min). Neither the BMG or the BDG conjugate was detected in the chromatograms of control tubes systemically run for that purpose. In addition, we demonstrated previously that hepatocytes killed by heating at 56° for 10 min [19] and then incubated as described above were unable to synthesize conjugated bilirubin. Moreover, in preliminary studies we evaluated, as follows, the possibility that some enzyme had leaked out of nonviable cells. A suspension of cells with low viability, as judged by Trypan blue staining (less than 80%), was centrifuged. After centrifugation, 1 ml of the supernatant fraction was incubated with 50 μM bilirubin, $MgCl_2$ and UDPGA for 15 min. Negligible amounts of conjugates (less than 1% of total bilirubin) were found in the chloroform extract submitted to thin-layer chromatography.

RESULTS

Rates of formation of BMG and BDG and their distribution between cells and supernatant fraction. The amounts of BMG and BDG synthesized by hepatocytes from normal rats incubated at a bilirubin substrate concentration of 50 μM were quantitated in cells and supernatant fractions at several time intervals (0, 2, 5, 15 and 25 min). No conjugates were detected at time 0, but both BMG and BDG were observed as early as after 2 min of incubation and then, as Fig. 2 shows, they increased up to 15 min of incubation. The figure also illustrates that both BMG and BDG contributed in similar proportions to conjugated bilirubin found in cells and supernatant fraction although a predominance of BMG was apparent; the amounts obtained in the fraction were much greater for both conjugates. The predominance of BMG coincided with a BDG decrease at 25 min of incubation, but the difference between the amounts of the two conjugates was statistically significant only in the supernatant fraction.

BMG and BDG formation at different bilirubin substrate concentrations in the incubation mixture. The results of these experiments are summarized in Table 1. It can be seen that hepatocytes from normal rats synthesized increasing amounts of BMG with the increase of bilirubin substrate concentration in the incubation mixture up to 50 μM where the capacity for glucuronidation became saturated for

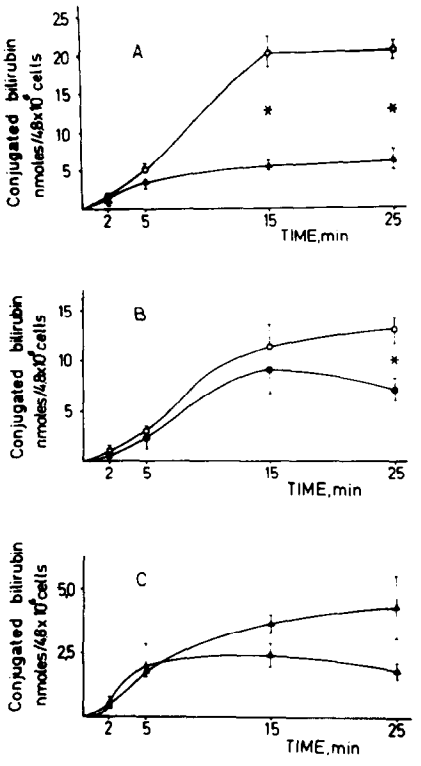

Fig. 2. Rate of formation of BMG and BDG by normal hepatocytes and their distribution between cells and supernatant fraction. The bilirubin substrate concentration in the incubation mixture was 50 μM. Incubations were carried out for 0 (N = 4), 2 (N = 2), 5 (N = 2), 15 (N = 4) and 25 (N = 3) min. No conjugates were detected at time 0. Panel A represents the time course of conjugated bilirubin (BMG + BDG) formation and its distribution between cells (△—△) and supernatant fraction (○—○). Panel B shows the amounts of BMG (○—○) and BDG (●—●) found in the supernatant fraction. Panel C refers to the amounts of BMG (△—△) and BDG (▲—▲) found in the pellet of cells. Each point is a mean value ± S.E.M. The asterisks (*) indicate differences that are statistically significant ($P < 0.05$).

Table 1. Effect of bilirubin substrate concentration in the incubation mixture on the formation of BMG and BDG by normal hepatocytes*

Bilirubin substrate (μM)	Conjugated bilirubin (nmoles/4.8×10^6 cells/15 min)	Glucuronides formed (% of conjugated bilirubin) BMG	BDG
11.3	1.9; 3.3† (2)	63; 66	37; 34
29.0	15.7 ± 0.8 (4)	63 ± 5	37 ± 5
50.0	26.4 ± 3.1 (4)	58 ± 5	42 ± 5
81.0	27.2 ± 3.5 (3)	64 ± 4	36 ± 4

* Data are mean values ± S.E.M. Conjugated bilirubin corresponds to the sum of BMG and BDG present in cells and supernatant fraction. The number of experiments is given in parentheses.
† Individual values.

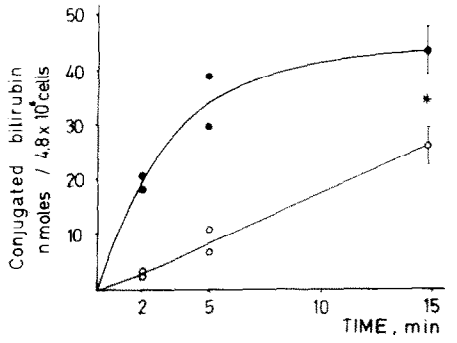

Fig. 3. Time course of conjugated bilirubin formation by hepatocytes from normal (○—○) and SP-treated rats (●—●). The bilirubin substrate concentration was 50 μM. For each group, incubations were carried out for 0 (N = 4), 2 (N = 2), 5 (N = 2) and 15 (N = 4) min. No conjugates were detected at time 0. The ordinate represents the sum of BMG + BDG determined in cells and supernatant fraction. Points at 2 and 5 min of incubation are individual values, and at 15 min of incubation they are mean values ± S.E.M. An asterisk (*) indicates a statistically significant difference ($P < 0.05$).

the fixed number of hepatocytes incubated. Conjugated bilirubin present in cells and supernatant fraction was mainly due to BMG for all the bilirubin substrate concentrations tested.

Effect of SP on BMG and BDG synthesis. The capacity for glucuronidation of hepatocytes from treated rats was examined at a bilirubin substrate concentration of 50 μM since at this concentration the capacity of controls reached the maximum. A more rapid rate of the formation of total conjugates (BMG and BDG quantitated in cells and supernatant fraction) was clear in the treated group in such a way that 58 ± 8% (N = 3) of total bilirubin determined in chloroform extracts was in the form of conjugated pigment after 15 min of incubation. This efficiency was significantly greater ($P < 0.05$) when compared to that of controls after a similar incubation period (35 ± 5%, N = 4). A tendency to reach a maximum constant value was observed earlier and at a higher level in the preparations of treated hepatocytes than in controls (Fig. 3). The greater capacity for glucuronidation of hepatocytes from treated animals was mainly due to BMG synthesis, whereas BDG synthesis, which was apparently also faster in these hepatocytes, became promptly saturated (Fig. 4). Most of the BMG synthesized by hepatocytes from SP-treated rats was found in the supernatant fraction.

DISCUSSION

Isolated hepatocytes may be a useful tool in the study of bilirubin glucuronidation and the effect of potential inducers of such a process. In the present study, the formation of BMG and BDG in isolated hepatocytes was examined. The polar derivatives of bilirubin were quantitated in cells and supernatant fractions by the alkaline methanolysis method [3]. The formation of mono- and diconjugates was only detected when UDPGA was added to the incubation system, and it was optimized as the level of this cofactor was artificially high [20]. As the level of endogenous UDPGA is low, it was suggested that the high level of this cofactor may favor the rate of glucuronidation in isolated hepatocytes [21].

Under the experimental conditions described in this paper, hepatocytes from normal rats initially formed approximately equal amounts of BMG and BDG. After 15 min of incubation when the amount of total bilirubin conjugates formed reached the maximum (Fig. 2A), a predomination of BMG was seen at different bilirubin substrate concentrations

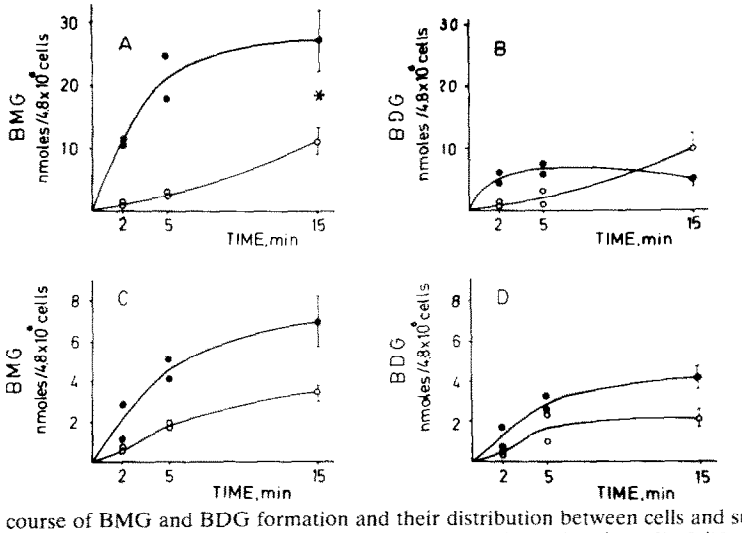

Fig. 4. Time course of BMG and BDG formation and their distribution between cells and supernatant fraction. For each group, BMG and BDG were quantitated for 0 (N = 4), 2 (N = 2), 5 (N = 2) and 15 (N = 4) min of incubation. Values for normal cells (○—○) and cells from treated rats (●—●) are individual experiments at 2 and 5 min of incubation, and at 15 min of incubation each point is a mean ± S.E.M. An asterisk (*) indicates a statistically significant difference ($P < 0.05$). Key: (A) BMG found in the supernatant fraction; (B) BDG found in the supernatant fraction; (C) BMG found in cells; and (D) BDG found in cells.

(Table 1). In addition, after a longer incubation period, the increase of BMG was coincident to a decrease of BDG (Fig. 2B). It has been shown by using microsomal preparations that, when the bilirubin substrate concentration was increased, proportionally more BMG was formed [7, 22]. However, by using liver slices it was observed that decreasing the bilirubin substrate concentration did not increase the proportion of BDG formed [10], as found in this study. It is accepted that a portion of BMG excreted in normal bile may be derived from hydrolysis of BDG and that β-glucuronidase may be involved in the mechanism of hepatic deconjugation [23]. However, it was reported that saccharo-1,4-lactone, a specific inhibitor of β-glucuronidase, did not increase the proportion of BDG synthesized by liver slices [10]. In our incubation system, the presence of 3 mM saccharo-1,4-lactone (undescribed experiments), a concentration lower than those used in rat liver slices, homogenates, or microsomes incubated with bilirubin [7, 10], impaired the stability of the preparation and cell viability, making the experiment unsuccessful. From our results we can assume that by increasing the bilirubin substrate concentration more BMG and BDG were proportionally formed until the capacity for glucuronidation became saturated for the fixed number of hepatocytes incubated. Since both conjugates mostly appeared in the supernatant fraction (Fig. 2), it is possible that saturating concentrations were reached intracellularly and that excretion was not limiting. This might favor the idea that conjugated bilirubin found in the supernatant fraction of isolated hepatocytes incubated with bilirubin may represent the biliary excretion process *in vivo* [24]. However, it is not possible to discard "leakage" of conjugates across plasma and canalicular membranes [10].

When hepatocytes from SP-treated rats were used, significantly greater amounts of conjugated bilirubin were formed (Fig. 3), mainly due to the more rapid synthesis of BMG (Fig. 4). Since at each incubation time the amount of BMG present in the supernatant fraction of treated cells exceeded that found in the supernatant fraction of controls, a more rapid release of BMG from treated cells to medium also may be suggested. These results are not fully in agreement with those reported previously in living rats and isolated rat livers where biliary excretion of bilirubin, increased by SP pretreatment, was produced at the expense of BDG [13, 14]. This probably indicates that conversion of BMG to BDG catalyzed by a glucuronyltransferase system is rate limiting in isolated hepatocytes. However, other possibilities are not excluded and non-enzymic conversion described when BMG was incubated in the presence of rat liver plasma membranes [12], or enzymic de-esterification of BDG induced by SP, may be involved in the results of this study.

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