JCI The Journal of Clinical Investigation

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*J Clin Invest.* 1988;82(2):608-616. https://doi.org/10.1172/JCI113639.

**Research Article**

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# Inhibition of Glutathione Efflux in the Perfused Rat Liver and Isolated Hepatocytes by Organic Anions and Bilirubin

## Kinetics, Sidedness, and Molecular Forms

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## Abstract

**Using isolated, in situ, single-pass perfused rat livers, incubations of freshly isolated hepatocytes, and sinusoidal membrane-enriched vesicles, we and others have shown the saturability of transport (efflux) of hepatic glutathione (GSH). These observations have implicated a carrier mechanism. Our present studies were designed to provide further evidence in support of a carrier mechanism for hepatic GSH efflux by demonstrating competition by liver-specific ligands which are taken up by hepatocytes. Perfusing livers with different substances, we found that: (*a*) sulfobromophthalein-GSH (BSP-GSH) had a dose-dependent and fully reversible inhibitory effect on GSH efflux, while GSH alone did not have any effect; (*b*) taurocholate had no inhibitory effect; (*c*) all of the organic anions studied, i.e., BSP, rose bengal, indocyanine green, and unconjugated bilirubin (UCB), manifested potent, dose-dependent inhibitory effects, with absence of toxic effects and complete reversibility of inhibition in the case of UCB. The inhibitory effects of UCB could be overcome partially by raising ($CoCl_2$-induced) hepatic GSH concentration.**

**Because of the physiological importance of UCB, we conducted a detailed study of its inhibitory kinetics in the isolated hepatocyte model in the range of circulating concentrations of UCB. Studies with $Cl^-$-free media, to inhibit the uptake of UCB by hepatocytes, showed that the inhibition of GSH efflux by UCB is apparently from inside the cell. This point was confirmed by showing that the inhibition is overcome only when bilirubin-loaded cells are cleared of bilirubin (incubation with 5% bovine serum albumin). Using Gunn rat hepatocytes and purified bilirubin mono- and diglucuronides, we found that both UCB and glucuronide forms of bilirubin inhibit GSH efflux in a dose-dependent manner. We conclude that the organic anions, although taken up by a mechanism independent of GSH, may competitively inhibit the carrier for GSH efflux from inside the hepatocyte.**

## Introduction

Glutathione exists in high concentrations (5–7 mM) in the liver, chiefly in the reduced form (GSH), and plays a major role in many detoxification and regulatory processes (1). The efflux of GSH from the liver is estimated to account for nearly all of hepatic GSH turnover (2). Using the isolated, perfused rat liver and freshly isolated hepatocyte systems, we have previously shown that sinusoidal efflux of GSH is saturable and thus, presumably, carrier mediated (3, 4). The saturability and transstimulation of GSH transport have been shown in sinusoidal membrane-enriched vesicles (5, 6).

In our initial work (3), we had conducted some preliminary experiments with sulfobromophthalein (BSP)[1]-GSH and had found that it could inhibit GSH efflux from the perfused rat liver. This outcome further supported the view that sinusoidal efflux of GSH is carrier mediated. Since BSP-GSH is an organic anion, we have examined the effect on GSH transport of several such molecules known to be taken up by hepatocytes. To accomplish this aim, we used the isolated, in situ, perfused rat liver system to screen organic anions (dyes), bilirubin, and taurocholate to find out whether they have any inhibitory effect on the GSH efflux. Since, among the compounds that exerted an inhibitory effect, bilirubin was the physiologically relevant and important organic anion, we studied the kinetics of its inhibitory effect on GSH efflux in more detail, using freshly isolated hepatocyte incubations. We extended our investigations to delineate whether the inhibitory effect is exerted from outside or inside the hepatocytes, as well as to identify the molecular form(s) of bilirubin (i.e., unconjugated vs. glucuronide forms) responsible for inhibition.

## Methods

*Chemicals and reagents.* GSH, unconjugated bilirubin (UCB), collagenase (type IV), bovine serum albumin, organic anion dyes (BSP, rose bengal, and indocyanine green) and Hepes were purchased from Sigma Chemical Co. (St. Louis, MO). HPLC-grade methanol was purchased from Fisher Scientific Co. (Springfield, NJ). All other materials used were of analytic reagent grade and were obtained from commercial sources.

*Animals.* Male Sprague-Dawley rats (Hilltop Laboratory Animals, Inc., Scottdale, PA), 200–300 g, were maintained on Purina rodent chow (Ralston Purina Co., St. Louis, MO) and water *ad libitum*. To induce an increased hepatic GSH concentration, some rats received $CoCl_2$ injections as described previously (3). The cumulative doses given in our present study were 80–100 mg of $CoCl_2$/kg.

Male hetero- and homozygote Gunn rats, 300–450 g, used in our studies were a generous gift of Dr. Anthony McDonagh of University of California, San Francisco, Liver Center. The distinct yellow color of the sera and the HPLC analysis of bile samples (see below), to establish the absence of bilirubin monoglucuronide (BMG) and bilirubin diglucuronide (BDG), were utilized as criteria for the acceptability of any homozygote Gunn rat used in our studies.

*Liver perfusions and hepatocyte incubations.* The design, method, and the apparatus described earlier (3) were used for our isolated, in

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*Received for publication 24 April 1987 and in revised form 14 March 1988.*

1. *Abbreviations used in this paper:* BMG and BDG, bilirubin mono- and diglucuronide; BSP, sulfobromophthalein; ECF, extracellular fluid; UCB, unconjugated bilirubin.

situ perfusions. The perfusion rates using oxygenated Krebs-Ringer bicarbonate buffer were 3.85±0.04 ml/min · g liver, perfusion pressures were 5.5±0.3 cm $H_2O$, and the $O_2$ uptakes were 2.13±0.03 μmol/min · g liver (mean±SEM, $n$ = 60–63). These values compare very closely to those reported in our earlier studies (3). We had also established that glutathione efflux from the perfused liver is virtually all in the form of GSH, i.e., reduced glutathione (3). Perfusions with $Cl^-$-free media were done with replacement of NaCl and KCl in the Krebs-buffer with equimolar sucrose. In all cases, glutathione S-transferase was measured in the perfusates, as before (3), and found to indicate no lysis.

Hepatocytes were isolated by the recirculating collagenase perfusion method of Moldeus et al. (7), and handled as described earlier (4). Cells were ≥ 93% viable at the onset of each incubation, as ascertained by 0.2% trypan blue exclusion and quantitation of extracellular fluid (ECF) glutathione S-transferase (4), a cytosolic enzyme marker. Incubations were routinely carried out with $10^6$ cells/ml (total initial volume = 10 ml). Our previous studies have established that practically all of glutathione effluxing from isolated hepatocytes is in the form of GSH. Additions of all compounds to be studied were made just before the suspension of hepatocytes to the Krebs-Henseleit buffer, supplemented with 12.5 mM Hepes at pH 7.4 and 37°C in 25-ml Erlenmeyer flasks. Thereafter, at the onset of each incubation, washed hepatocytes were suspended and rapidly dispersed in the incubation media. The flasks containing cell suspensions were quickly returned to the heating shaker bath. About 1–2 min was allowed for temperature equilibration and settlement of any transient disturbances, after which the first sample was taken and expressed as $t$ = 0 sample. Incubations were done under room air with gentle shaking and periodic samples of cell suspension (1 ml) were removed for immediate separation of cells and ECF (5-s centrifugation on Beckman Instruments Microfuge) for subsequent assays and analyses. Cell lysis was measured during the course of incubation by the measurement of released glutathione S-transferase and the results were used to correct the ECF data for contributions made by cytosolic contents due to lysis. The cumulative lysis throughout our incubations generally remained well below 10%. Certain control incubations were carried out in the absence of hepatocytes (cell-free incubations), or with hepatocytes but at ≤ 4°C, as described in Results. $Cl^-$-free incubations were done using an equimolar sucrose-Hepes buffer. In incubations with UCB, BMG, and BDG, bovine serum albumin was also added to the incubation media, just prior to the addition of these compounds. The initial molar ratio of bilirubin moieties to albumin was set at ~ 1. Detectable effects on the ECF GSH were observable only at albumin concentrations ≥ 0.5% (≥ 75 μM). However, this effect is due to the promotion of mixed disulfide formation in response to elevated albumin levels (unpublished observations). For our present studies, all incubations were done with < 0.5% albumin.

After complete removal of the supernatants of centrifuged incubation samples, the cell pellets ($10^6$ cells) were ordinarily resuspended in buffer (0.5 ml) and cellular GSH was extracted with addition of 10% trichloroacetic acid (1:1, vol/vol), as described before (4). Whenever cellular bilirubin measurements were required, the pellets were extracted with 0.5 ml of HPLC-grade methanol (Fisher Scientific Co.). Our trials established that a single extraction step with 0.5 ml of methanol could recover 80% of the total cellular unconjugated and glucuronide forms of bilirubin that could be recovered with a total of four successive extractions with methanol (levels of bilirubin in additional washes fell below the detection levels of our methods). This one-step extraction efficiency, i.e. 80%, was the same for the recovery of cellular GSH content by 0.5-ml methanol extraction, as compared to buffer + trichloroacetic acid extraction. Thus, in extractions of cell pellets with methanol, we used a correction factor (multiplication by 1/0.8 = 1.25) to estimate the true cellular values, based on an 80% efficiency of extraction.

In our studies, perfused livers from ad lib-fed rats of different groups had average GSH concentrations in the typical range of 5–6 μmol/g. Isolated hepatocytes averaged at ~ 40 nmol/$10^6$ cells or ~ 4 μmol/g liver of GSH ($10^8$ cells = 1 g), except for the $CoCl_2$-treated group which reached an average value of ~ 7 μmol/g. Mean±SEM for each group is presented in the tables and the legends to the figures.

To measure the total glutathione S-transferase content of the cells used in any incubation, additional samples of $10^6$ pelleted cells were resuspended in 0.5 ml of buffer and digested with 10% triton X-100 (1:1, vol/vol). All resuspended pellets treated with trichloroacetic acid, methanol, or Triton X-100 were again centrifuged in a Microfuge and the supernatants were used for subsequent analyses. The cumulative lysis during 1-h incubations usually was well below 10% of total cell population. The values of GSH in the medium were corrected for the contribution by lysis, as before (4).

*Assays.* GSH in perfusates and incubation media (ECF) were measured by the recycling assay of Tietze (8). Whenever addition of other compounds (i.e., organic anion dyes, bile acids and bilirubin in conjugated or unconjugated form) was made, we ascertained that their presence had no effect on determination of GSH values under our assay conditions. Hepatic GSH was measured by the Owens and Belcher (9) method, as described previously (3). Perfused liver homogenates, or resuspended cell pellets were precipitated with 10% trichloroacetic acid (1:1 vol/vol) and the supernatants were assayed for GSH. Glutathione S-transferase was measured by the formation of the conjugate of 1-chloro-2,4-dinitrobenzene-GSH conjugate, as before (3, 4).

Organic anion dyes were measured by direct spectrophotometric assays after scanning for determination of their peak absorbance. Thus, BSP and BSP-GSH conjugate (the latter prepared by the method described by Whelan et al. [10]) were assayed at 580 nm (under alkaline conditions), rose bengal at 550 nm, and indocyanine green at 800 nm.

*Bilirubin solutions and HPLC analysis.* All preparations, purifications, handling, and use in perfusions or incubations of UCB and BMG were done under very dim lighting. Intermediate storage of compounds was under total darkness at −16°C. In addition, freshly purified BMG and BDG were stored under 99.99% argon at −16°C for no longer than overnight, before use.

Fresh stock solution of UCB (5 mM in 100 mM $Na_2CO_3$) was prepared, according to the method described by Paumgartner and Reichen (11), just before use in perfusions or incubations. The stock solution was then diluted to the desired concentration to obtain a molar ratio of UCB:bovine serum albumin of 1:1. Control incubations with the highest concentration of $Na_2CO_3$ alone (without bilirubin), used in our experiments, showed no effect on the GSH efflux or any other parameter.

To obtain BMG, we used the preparative gradient HPLC method described by Spivak and Carey (12). First, to obtain BMG- and BDG-rich bile, we infused 50 μl/min of 1 mM UCB (diluted in 0.9% NaCl from the 5 mM stock solution described above) i.v., through a jugular catheter, into Sprague-Dawley rats and collected bile, in near darkness, on ice, under a stream of 99.99% pure argon. Proteins of the bile sample were denatured and precipitated by a 3:1, vol/vol, treatment with HPLC-grade methanol. 1 ml of the supernatant was injected onto a preparative column (Altex Ultrasphere-ODS, reverse phase, 5 μm, 10 mm ID × 25 cm L; Altex Scientific, Berkeley, CA) for separation by the gradient method of Spivak and Carey (12) using a flow rate of 3.5 ml/min. The buffers used for HPLC were gassed with the high-purity argon. BDG fraction eluted with a retention time of 8–9 min and BMG fraction with a retention time of 13–14 min. These fractions were collected in three equal lots (~ 5 ml each) and concentrated down (to ≤ 0.5 ml each) under a stream of high-purity argon at room temperature. The concentrated samples were subsequently desalted by Sep-Pak $C_{18}$ cartridges (Waters Associates, Milford, MA), with all steps carried out using ice-cold, argon-gassed double-distilled water and HPLC-grade methanol as follows. Each cartridge was wetted by 5 ml of water. The concentrated sample was loaded onto the cartridge and washed with 3 ml of water. Thereafter, BMG or BDG was eluted by 2–3 ml of methanol. These desalted samples were evaporated down to near dryness under a high-purity argon stream at room temperature and stored overnight at −16°C. On the following morning, the specimens were

taken to complete dryness to eliminate any traces of methanol. Thereafter, BMG or BDG were resuspended at the desired concentration in the incubation media containing bovine serum albumin just before the addition of the hepatocytes, as described earlier.

To analyze the perfusate, or incubation media (ECF) samples, for UCB, BMG, and BDG, a fivefold (vol/vol) dilution of each sample was made with HPLC-grade methanol to precipitate the proteins, in preparation for loading the sample onto the analytical column. Cell-associated bilirubin was extracted with 0.5 ml HPLC-grade methanol, as stated before, and after centrifugation (5 s on a Microfuge), the supernatant was injected onto the column. The analytical column was an Altex Ultrasil-ODS, reverse-phase column (10 μm, 4.6 mm ID × 25 cm L). The retention times with 1 ml/min flow rate for the different peaks were: BDG, 12.5 min; BMG, 17 min; and UCB, 25 min. With our system, samples with concentrations as low as 1 μM were reliably quantifiable using a 1-ml injection of 1:5 methanol-precipitated sample and an absorbance scale of 0.02 at 455 nm. Standards of UCB were run to quantitate all forms of bilirubin, based on the reported identity of the extinction coefficients of the different moieties (13).

## Results

### *Inhibition of sinusoidal GSH efflux from the perfused liver by BSP-GSH*

We had previously reported the ability of ~ 10 μM of BSP-GSH to inhibit (by ~ 30%) the sinusoidal GSH efflux from the isolated, perfused rat liver (3). In our present studies, we first extended our observations over a range of BSP-GSH concentrations to establish whether there is a dose dependence between the inhibitor concentration and GSH efflux. In these perfusions, after an initial control period, a 30-min step infusion of BSP-GSH was given (data not shown; refer to Fig. 1 showing the design and data from similar studies using UCB). About 15 min after the onset of the infusion, the declining rate of GSH efflux reached a near plateau. Later, when the BSP-GSH infusion was stopped, the efflux recovered in about 15 min to control (pre-BSP-GSH) levels. Thus, the inhibitory effect of BSP-GSH was completely reversible. There were also no adverse effects manifested throughout these perfusions as evidenced by the $O_2$ uptake values, which remained at typical control levels (3); absence of breakdown of the liver, as monitored by the release of GSH S-transferase in the perfusates; and steadiness of pressure throughout each perfusion.

We used the average value of the data points defining the plateau value of inhibited efflux (15–30 min after onset of infusion) to compute and express the inhibition of GSH efflux as the percentage of the mean control (uninhibited) efflux. The results of a number of perfusions conducted with different doses of BSP-GSH revealed a dose dependence between the inhibition of GSH efflux and perfusate BSP-GSH concentration (Table I). The corresponding rates of uptake of BSP-GSH by livers were measured by the difference in the inflow–outflow concentrations of BSP-GSH in the perfusates during the plateau phase of inhibition (Table I). The concentration of BSP-GSH in the effluent perfusate fell to undetectable levels in less than 2 min after the cessation of its infusion into the perfusion line.

### *Effect of bile acids and organic anion dyes on sinusoidal GSH efflux from the perfused liver*

Taurocholate, at the range of concentrations tested, i.e., 5–100 μM ($n$ = 3), did not exert any inhibitory effect on the efflux of GSH. On the other hand, all of the organic anion dyes tested in two to three separate perfusions exerted potent inhibitory effects on the GSH efflux with 10–15 μM BSP exerting 30–50% inhibition, 4 μM rose bengal inhibiting by 50%, and 1.5–2.0 μM indocyanine green inhibiting by 40–45%. The inhibitory effect of these three dyes was accompanied by toxic manifestations, such as declining $O_2$ uptake, progressively increasing pressure (resistance to flow) during the infusion of the dye, and incomplete recovery of the GSH efflux to control levels following the cessation of the infusion of these compounds (data not shown).

*Table I. Inhibition of GSH Efflux from the Perfused Rat Liver by BSP-GSH**‡*

| Perfusate [BSP-GSH] | Rate of BSP-GSH uptake | Percent inhibition of GSH efflux§ | $n$‖ |
|---|---|---|---|
| μM | nmol/min · g liver | | |
| 4.9±0.3 | 9.0±0.5 | 18.9±3.7¶ | 4 |
| 10.0±0.7 | 11.1±0.8 | 28.5±2.7 | 11 |
| 31.7±1.1 | 20.0±4.7 | 49.6±2.2 | 5 |

Values given as mean±SEM.
* See text for details of design.
‡ The livers were from normal, *ad lib*-fed rats with 5.3 ± 0.4 μmol GSH per g liver.
§ % Inhibition (from control efflux) was calculated using the mean of data points in the 15–30 min period after the onset of BSP-GSH infusion.
‖ $n$, number of perfusions.
¶ The rate of control (uninhibited) efflux for the total number of livers used in this study was 15.2±0.9 nmol GSH/min · g liver.

### *Inhibition of sinusoidal GSH efflux from the perfused liver by bilirubin and the effect of hepatic GSH concentration*

Since bilirubin is a physiologically relevant and important organic anion, we were interested in determining if, in the range of normal plasma concentrations of UCB (≤ 20 μM), it would exert any inhibitory effect on the GSH efflux from the perfused liver. UCB caused an inhibition of the GSH efflux which was not accompanied by the toxic manifestations observed with the dyes. In fact, it was possible to conduct perfusions with two successive and increasing doses of UCB on the same liver, a sample of which is presented in Fig. 1. As can be seen, after each step infusion of UCB, the GSH efflux arrived at a near plateau in 15 min, after the onset of each respective dose of infusion.

A group of perfusions was done on livers with normal hepatic GSH levels, as well as a group with elevated hepatic GSH levels ($CoCl_2$-induced) to determine if raising intracellular GSH would overcome the inhibitory effect of UCB (presumably by competition for the carrier). As seen in Table II, there was a dose-dependent inhibition of GSH efflux. In addition, significantly less inhibition of GSH efflux was observed in livers with elevated GSH compared to the livers with normal GSH. The rate of uptake of UCB increased with increasing concentrations in the perfusate, but was indistinguishable between normal and $CoCl_2$-treated groups. Normal and $CoCl_2$-treated livers had indistinguishable GSH effluxes due to both values being near saturation levels (pooled value of efflux shown in the footnote to Table II). Thus, apparently, elevated

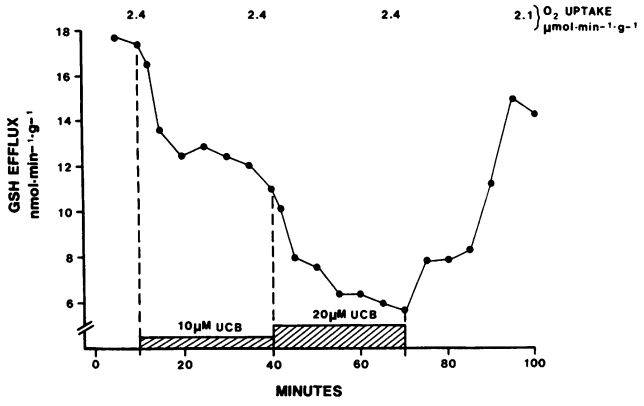


*Figure 1.* Sample perfusion with bilirubin. Results from perfusion of one liver with two successively increasing doses of UCB, i.e., 10 and 20 μM, are shown. Shaded areas highlight the duration of perfusion for each dose. $O_2$ uptake at the end of each interval is shown on top.

hepatic GSH did not overcome inhibition by a greater absolute rate of efflux of GSH, or a decreased rate of uptake of UCB.

### *Kinetics of the inhibition by bilirubin of GSH efflux from freshly isolated hepatocytes and the effect of cellular GSH concentration*

To extend our findings with UCB in the perfused liver, and to study the kinetics and characteristics of inhibition in more detail, we turned to the isolated cell system. The results from a sample experiment are shown in Fig. 2, which also presents the design of these incubation experiments. As before (4, 14), the appearance of GSH in the incubation medium (referred to as ECF) was nearly linear and accompanied by a concurrent drop in the cellular GSH, such that the total (cells + ECF) GSH remained near-constant, or decreased slightly, in 30–60-min incubations. Thus, as before (4, 14), these hepatocytes were

*Table II. Inhibition of GSH Efflux from Rat Livers Perfused with UCB**

| Perfusate [UCB] | Rate of UCB uptake | Percent inhibition of GSH efflux‡ | | |
|---|---|---|---|---|
| | | Normal | $CoCl_2$-treated | *n* |
| *μM* | *nmol/min · g liver* | | | |
| 5.8 | 9.4 | 7.0 | — | 1 |
| 11.1, 12.6 | 28, 28 | 28, 42 | — | 2 |
| 21.0±1.5 | 37.9±1.9 | 59.9±2.7 | — | 7 |
| 21.0±0.9 | 37.3±6.9 | — | 43.7±0.9§ | 3 |

* Liver GSH values were 6.0±0.2 (mean±SEM, $n$ = 10) and 11.1±0.6 ($n$ = 3) μmol/g liver for normal and $CoCl_2$-treated groups, respectively.

‡ The rate of control (uninhibited) efflux for the normal vs. $CoCl_2$-treated groups were similar and their pooled value was 15.4±0.6 nmol GSH/min · g liver. The percent inhibition (from control efflux) was calculated using the data from the 15–30-min period after the onset of bilirubin perfusion.

§ The mean percent inhibition for $CoCl_2$-treated group (i.e., 43.7) was significantly lower ($P < 0.01$, unpaired $t$ test) than the corresponding normal group (i.e., 59.9) perfused with the same concentration of UCB.

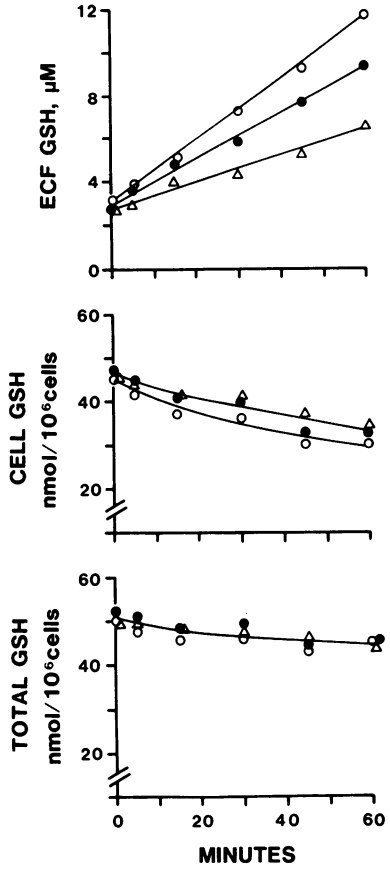


*Figure 2.* Sample incubations of hepatocytes showing the dose-dependent inhibitory effect of UCB added to the incubation medium. (○) Control (no UCB); (●) 1 μM UCB; (△) 5 μM UCB. The rates of control and inhibited efflux were calculated (linear regression) from the slope of the accumulation of ECF GSH (*upper panel*). The inhibition of GSH efflux is not due to depleted cellular GSH (*middle panel*). As before (4), the hepatocytes were not synthesizing any GSH *de novo*, such that the sum of ECF and cell GSH did not rise during the incubations (*lower panel*).

not synthesizing any net amount of GSH *de novo* during our incubations, and the decline of cellular GSH was almost quantitatively accounted for by the loss due to efflux. The rate of efflux in each incubation was estimated with the slope of the linear regression fit to the ECF GSH data, as shown in Fig. 2. As can be seen, doses as low as 1 and 5 μM ECF UCB were capable of inhibiting the GSH efflux in comparison to control. We have verified that the fall in the rate of GSH efflux due to UCB was not accompanied by a fall in the cellular ATP levels (Fernandez-Checa et al., manuscript submitted for publication) or generally reduced transport functions, as checked with [$^{14}$C]glycine uptake (data not shown).

Fig. 3 presents the bilirubin data from a group of incubation experiments with 20 μM ECF UCB. Shortly after the suspension of hepatocytes in the medium containing 20 μM UCB (~ 2 min to the onset of $t$ = 0 for our kinetic measurements), there was a considerable initial drop (~ 25%) in the ECF UCB. This drop was present, even at 4°C incubation and in ECF samples taken as quickly as it was feasible, i.e., a few seconds, after the suspension of cells. However, whereas in 37°C incubations there was a further continuous decline in the ECF UCB to concentrations below 10 μM at 60 min, there was no further fall in the 4°C incubations. Cell-free control incubations conducted in parallel at 37°C showed no spontaneous decline of ECF UCB; thus, post zero-time disappearance of ECF UCB was caused by the presence of cells due to continued binding/uptake of UCB. Cell UCB values (Fig. 3) showed the presence of a large early component, correlated with the initial rapid fall observed in the ECF UCB. This high, near-instantaneous, cell-associated value is probably due to the binding of bilirubin to the cell surface and exterior of the plasma mem-

brane of hepatocytes. Thus, the cell-associated UCB values at any time point represent a combination of a large membrane-bound component and the intracellular UCB. Therefore, at any given time point, it is not possible to accurately determine the fraction of cell-associated UCB that is truly intracellular. Whereas cell UCB accumulated to a constant level in 4°C incubations, in 37°C incubations it reached an early peak and subsequently declined. The additional decline was presumably due to conjugation and metabolism of UCB to BMG (and BDG) as shown in Fig. 3. Cell BMG also rose to a peak and declined in 37°C incubations, while it remained undetectable in 4°C incubations, consistent with the apparent lack of metabolism manifested by the ECF and cell UCB data. The destination of declining cell BMG was not known, since the appearance of cell BDG could not account for it (data not shown) and the amount of BMG or BDG released to the medium was below detection levels which precluded their accurate estimation. Thus, ~ 15% and ~ 25% of the total bilirubin added at the beginning of each incubation could not be accounted for at 30 and 60 min, respectively. We do not know whether progressive binding of bilirubin to membranes with subsequent difficulty of extraction contributes to poorer recoveries, or not.

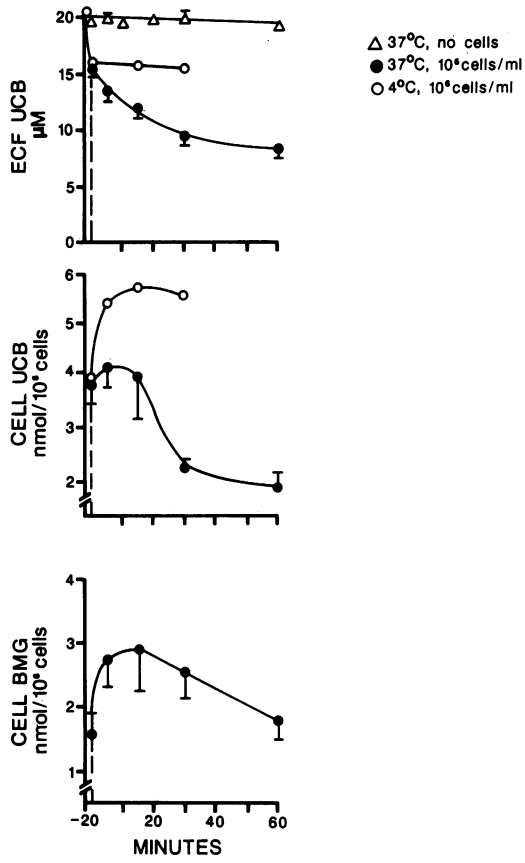


*Figure 3.* The fate of bilirubin in incubations with hepatocytes from Sprague-Dawley rats. UCB was added to the incubation media (ECF) containing equimolar bovine serum albumin. Thereafter, hepatocytes were rapidly dispersed in ECF. There was a precipitous initial fall in ECF UCB after suspension of hepatocytes as observed by the zero time sample, identified by vertical dashed line, taken ~ 2 min after cell dispersion. This fall, which was present in ECF samples taken as rapidly as possible (few seconds) after dispersing the cells, represents a large binding component of UCB to the cell plasma membranes. This early component that is observed also in the cell UCB data was present even at 4°C incubations (○). However, only at 37°C incubations (● with bars, representing mean±SEM of $n$ = 3–8 incubations), the fall in the ECF UCB continued beyond the zero time, and was accompanied by the concurrent appearance and metabolism of cell UCB to BMG (and BDG, not shown). The uptake and metabolism at 4°C incubations were absent after the initial binding phase. There was no spontaneous fall in ECF UCB in cell-free incubations (△, $n$ = 1–3).

A number of cell incubations were done as above, at 37°C and in the presence of different initial concentrations of ECF UCB, to define the kinetics of inhibition by UCB of GSH efflux. These studies were done with hepatocytes from normal, ad lib-fed rats, as well as those from rats treated with $CoCl_2$, to raise their hepatocellular GSH. The results are summarized in Fig. 4. The data for inhibited GSH efflux, expressed as the percentage of control, clearly defined a dose-dependent relationship between the degree of inhibition and the initial ECF concentration of the inhibitor. In addition, as was the case in the perfused liver studies, the inhibition was partially overcome by increased cellular GSH concentration. However, changes in cellular GSH were not accompanied by changes in the uptake of UCB as measured by the extracellular disappearance and cellular appearance (data not shown). Since raising GSH overcame the inhibition, it appeared that UCB competitively inhibited GSH transport.

### *General characteristics of the inhibition by bilirubin of GSH efflux from isolated hepatocytes*

Up to this point, we had observed the inhibitory effect of UCB after its addition to the incubation medium. Since UCB is taken up by the hepatocytes and conjugated to the mono-(BMG) as well as diglucuronide (BDG) forms, two other important questions remained to be explored. These were: (*a*) Does bilirubin have to be taken up to exert its inhibitory effect from inside the cell? (*b*) If so, is it the unconjugated

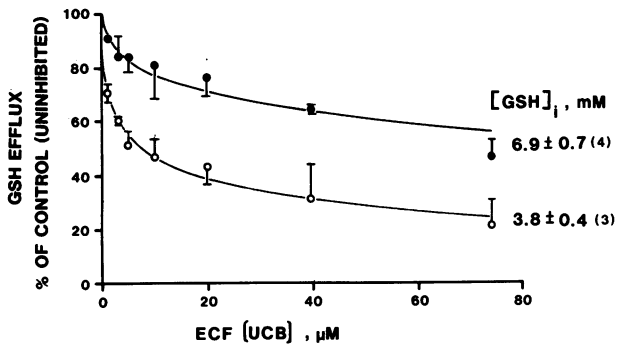


*Figure 4.* Kinetics of inhibition of GSH efflux from Sprague-Dawley hepatocytes in response to the addition of UCB to the incubation medium and the effect of cellular GSH. The data (mean±SEM, $n$ = 3–4), expressed in units of GSH efflux as percentage of control, or uninhibited efflux, show a dose-dependent relationship between the initial ECF concentration of UCB and the magnitude of inhibition. Raising the cellular GSH concentration, denoted as $[GSH]_i$, from a mean of 3.8 to 6.9 mM caused the inhibition to be overcome partially. This effect was not due to changes in UCB uptake (not shown), or increased GSH efflux (since both cell GSH concentrations are associated with comparable efflux rates, pooled value = 0.180±0.015 nmol GSH/min per $10^6$ cells, due to being near or at saturation [3, 4]). The continuous curves are the result of a model-fitting exercise presented in the Discussion.

(UCB) or conjugated (BMG and BDG) form of bilirubin that inhibits GSH efflux? To attempt to answer the above two questions dealing with the issues of sidedness and molecular form, we did the following experiments.

*Sidedness.* Evidence that UCB had to be taken up by the hepatocyte in order to inhibit the cellular GSH efflux was obtained with the following approach. Wolkoff et al. (15) have shown that the uptake of organic anions, including bilirubin, is dependent on the presence of $Cl^-$ ions in the incubation media, or perfusates, in short-term rat hepatocyte cultures, or perfused livers. Taking advantage of this observation, we incubated freshly isolated hepatocytes in the presence and absence of UCB in Krebs-Hepes (control) and $Cl^-$-free (sucrose-Hepes) media (see Methods). Table III shows the summary of the results from cell incubation experiments. Identical cellular GSH effluxes occurred in Krebs and $Cl^-$-free media, in the absence of UCB. However, when UCB was present, only the GSH efflux from cells in Krebs media was inhibited. No inhibition of the GSH efflux was observed in the $Cl^-$-free medium. Experiments with perfused livers corroborated these findings, i.e., no inhibitory effect of UCB was observed when livers were perfused with 10–20 $\mu$M UCB with $Cl^-$-free medium (data not shown). It should also be noted that the initial cell surface binding in $Cl^-$-free medium and Krebs media were identical (not shown). However, the progressive decline in medium UCB and appearance of cellular BMG were not observed in the $Cl^-$-free medium. This also indicates that surface binding alone does not inhibit GSH efflux.

An additional study was conducted to determine whether the loading and unloading of hepatocytes with UCB would show a clear-cut relationship to GSH efflux. Thus, hepatocytes were preincubated in Krebs medium with and without 20 $\mu$M UCB for 30 min, as before. However, subsequently, they were washed and resuspended in Krebs media with 0.1% and 5% bovine serum albumin for 20 min. This was done to remove cellular UCB with the high concentration of acceptor albumin in the medium. Afterwards, cells were washed again, resuspended in Krebs buffer, and incubated for 30 min to determine the rate of GSH efflux. After incubation with 5% albumin, no UCB remained in the cells whereas 0.1% albumin was ineffective in removing cellular bilirubin. Table IV shows the summary of the results from these experiments. As can be seen, the GSH efflux from cells incubated with 5% albumin recovered to levels indistinguishable from control. However, cells resuspended in albumin-free medium, retained an inhibited level of GSH efflux (i.e., 41% of control) in the total absence of any extracellular UCB. This degree of inhibition was close to the original level of inhibition measured in the presence of 20 $\mu$M extracellular UCB (i.e., 44% of control).

*Molecular form.* To delineate whether it is the unconjugated or the conjugated form of bilirubin that is responsible for inhibition of GSH efflux, we did the following set of studies.

To clarify whether UCB alone can exert an inhibitory effect, we used hepatocytes obtained from Gunn rats. Hetero- and homozygote Gunn rat hepatocytes were incubated in the same way as those from Sprague-Dawley rats, described above. Table V presents the results of these experiments. In both groups of cells (homozygote and heterozygote) addition of UCB caused the inhibition (dose-dependent) of GSH efflux from hepatocytes. However, while heterozygote cells were producing BMG and BDG during the incubation, there were no detectable traces of BMG (and BDG) in the cells from homozygote Gunn rats (not shown). Thus, it was clear that UCB alone, without being converted to BMG and BDG, could inhibit the GSH efflux.

After delineation of this point, the possibility still remained that BMG and BDG were also capable of inhibiting the GSH efflux. Therefore, we performed a group of experiments using BMG and BDG purified by the preparative HPLC method of Spivak and Carey (12), as described in Methods. The effect of BMG was studied more extensively than the BDG in an attempt to define its kinetics of inhibition. The disappearance of ECF BMG in 1-h incubations was similar to that observed with UCB in cells from Sprague-Dawley and Gunn rats (not shown), with negligible spontaneous disappearance of ECF BMG in cell-free incubations. Also the main difference from UCB data was that the initial, rapid fall of ECF BMG was only a small fraction of that of UCB. The fractional inhibition of GSH efflux by BMG was indistinguishable among the different cell types, i.e., Sprague-Dawley, heterozygote and homozygote Gunn rats. Hence, the data showing the inhibitory kinetics for all cell types have been pooled and presented in Fig. 5. As can be seen, there was a dose-dependent inhibition of GSH efflux by BMG, similar to that observed with UCB.

The effect of BDG was verified only for initial ECF BDG of 2.21±0.2 uM in 5 cell preparations and found to decrease the hepatocellular GSH efflux to 57.2±6.3% of control (uninhibited) efflux.

## Discussion

Using isolated, in situ, single-pass rat liver perfusions and freshly isolated hepatocyte incubations, we have reported essentially identical kinetics of sinusoidal GSH efflux as a func-

*Table III. Effect of UCB in $Cl^-$-free (Sucrose-Hepes) Medium on GSH Efflux from Isolated Hepatocytes*

| Incubation ($n$ = 3) | Cellular [GSH] | | GSH efflux | GSH efflux | $P$* |
|---|---|---|---|---|---|
| | 0 min | 60 min | | | |
| | *nmol/$10^6$ cells* | | *nmol/min per $10^6$ cells* | *% control* | |
| Krebs-Hepes | 38.8±1.1‡ | 28.5±8.6 | 0.180±0.05 | 100 | — |
| Sucrose-Hepes | 35.8±1.2 | 22.2±4.1 | 0.190±0.06 | 106 | NS |
| 20 $\mu$M BR in Krebs-Hepes | 40.7±4.0 | 33.6±5.4 | 0.062±0.03 | 34 | <0.01 |
| 20 $\mu$M BR in sucrose-Hepes | 38.5±1.4 | 23.4±4.9 | 0.179±0.06 | 99 | NS |

* $P$ values, from unpaired $t$ tests, are for the GSH efflux in comparison to Krebs-Hepes control. ‡ Mean±SD, $n$ = 3.

*Table IV. Effect of Bilirubin Loading and Unloading of Hepatocytes on GSH Efflux**

| Incubation | Cellular [GSH] 0 min | Cellular [GSH] 30 min | GSH Efflux | Cellular UCB | GSH efflux | $P^{\ddagger}$ |
|---|---|---|---|---|---|---|
| | *nmol/$10^6$ cells* | | *nmol/min per $10^6$ cells* | *nmol/$10^6$ cells* | *% control* | |
| During loading | | | | | | |
| Control | 54.0±1.0[§] | 39.2±0.5 | 0.223±0.021 | | 100 | |
| 20 μM UCB | 53.0±0.9 | 48.2±2.0 | 0.099±0.021 | 4.2±0.7 | 44 | <0.005 |
| After unloading | | | | | | |
| Control | 29.7±1.1 | 27.6±2.7 | 0.207±0.040 | | 100 | |
| 0.1% BSA | 31.8±2.2 | 27.4±3.6 | 0.085±0.012 | 3.6±0.3 | 41 | <0.01 |
| 5% BSA | 27.3±0.5 | 25.9±0.4 | 0.174±0.030 | <0.1 | 84 | N.S. |

* Hepatocytes were preincubated with and without 20 μM UCB for 30 min to load some of the cells with bilirubin, while measuring their GSH efflux. Subsequently, these preincubated cells (both control and UCB-incubated) were washed and suspended in media containing 0.1% and 5% BSA for an additional 20 min. Finally, the cells were washed again and resuspended in Krebs-Hepes medium with no extracellular UCB, or BSA, to measure their GSH efflux over a 30-min interval (after unloading, above). Separate measurements established that the incubation of hepatocytes in 5% BSA alone does not affect the rate of GSH efflux after the cells are washed of BSA (data not shown). ‡ *P* values, from unpaired *t* tests, are for the GSH efflux in comparison to control for each set of data. § Mean±SD, n = 3.

tion of hepatocellular GSH concentration in both systems (3, 4). The kinetics of efflux was characterized by a sigmoidal-saturable shape, which could not be fitted by the Michaelis-Menten model. The Hill equation was used, chiefly as a utilitarian model, to fit the data from perfused livers and isolated hepatocyte studies to extract the $V_{max}$ (20–26 nmol·min$^{-1}$·g$^{-1}$) and $K_m$ (3.2–3.6 μmol/g or mM) values for the transport kinetics. A value of $n \sim 3$ was required as the number of cooperative binding/transport sites in the Hill model to obtain the best fits (3, 4). Thus, the saturability of the transport rate implicated the possible involvement of a carrier mechanism. However, saturability is a necessary, but not a sufficient criterion for carrier-mediated processes. Two additional qualifying criteria were verified and met subsequently. One, i.e. transstimulation, was demonstrated in the sinusoidal membrane-enriched vesicle model (6). The other, the ability of other but similar molecules to compete with and inhibit the transport of GSH, was confirmed with BSP-GSH in our preliminary work (3).

*Table V. Effect of UCB on the GSH Efflux from Gunn Rat Hepatocytes**

| | Initial ECF [UCB] | Cell BMG | GSH efflux |
|---|---|---|---|
| | *μM* | | *% control* |
| Heterozygote | 20.1±1.1 (4)[‡] | + | 39.9±3.7 |
| Homozygote | 4.3 | − | 82.1 |
| | 8.5 | − | 64.3 |
| | 20.4±1.6 (3) | − | 50.4±5.6[§] |

* The hepatocytes used in these studies had 42.2±2.9 nmol GSH/$10^6$ cells and a control (uninhibited) efflux of 0.172±0.029 nmol GSH/min per $10^6$ cells ($n$ = 11, preparations from four heterozygote and three homozygote Gunn rats).
‡ Mean±SEM ($n$).
§ Not significantly different from the corresponding value for the heterozygote group.

In our present work, we have attempted to explore the generality of the inhibitory phenomenon by extending our observations to other organic anions. We have found that a bile acid transported by a carrier mechanism, i.e., taurocholate (studied up to 100 μM), had no effect on the sinusoidal GSH efflux from the perfused liver. In contrast, all of the organic anions screened, i.e., BSP, rose bengal, indocyanine green, and UCB, at rather low concentrations, i.e., 2–20 μM, were able to inhibit the sinusoidal GSH efflux from the perfused rat liver. However, the effect of all of the tested organic anions, with the exception of UCB, was accompanied by toxic manifestations, i.e., declining hepatic $O_2$ uptake, increasing perfusion pressure (resistance to flow), and incomplete recovery of GSH efflux to pre-organic anion infusion (control or uninhibited) values.

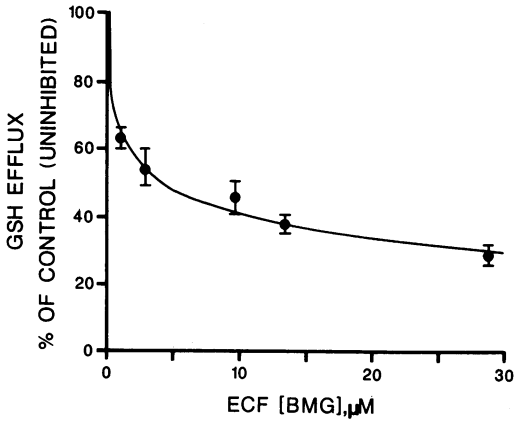


*Figure 5.* Kinetics of inhibition of GSH efflux by BMG. No differences were observed among the hepatocytes from Sprague-Dawley, heterozygote and homozygote Gunn rat cells; therefore, the data (mean±SEM, $n$ = 3–8) were pooled for presentation. The inhibition, similar to the incubations with UCB, was dose-dependent. Cellular GSH concentrations were also indistinguishable between the different groups, pooled value = 39.2±2.5 ($n$ = 25). The continuous curve represents the result of the model-fitting exercise presented in the Discussion.

Since bilirubin is a physiologically relevant and important organic anion, we extended our study of its detailed kinetics of inhibition of GSH efflux using the freshly isolated hepatocyte system. Incubations of the hepatocytes with physiological circulating levels of UCB, i.e., $\leq 20$ $\mu$M, resulted in potent, dose-dependent inhibitory effects (up to $\sim$ 70% inhibition) that could be partially overcome by raising the cellular GSH concentrations. (Note: We do not know whether the mechanism by which the cellular GSH is raised will have effects on the outcome of our studies. $CoCl_2$ treatment does not affect the magnitude of sinusoidal GSH efflux, which remains at maximal saturation levels. Thus, it presumably increases the hepatic GSH pool size by increasing the synthetic rate.) We also ruled out the possibility that falling cellular ATP levels were the cause of falling GSH efflux in response to UCB, and verified that UCB had no effect on the uptake of [$^{14}$C]glycine during our incubations (up to 1 h). Thus, these observations were consistent with the view of UCB competitively inhibiting the GSH carrier. It should be noted that in isolated cells an uncertain but significant portion of the GSH efflux could represent the canalicular equivalent. Substantial canalicular GSH efflux occurs in the intact liver (16), and some organic anions can inhibit this component (17). BSP-GSH does not affect canalicular GSH transport (17, 18). However, the effects of UCB and glucuronides have not been examined. Therefore, the effects of bilirubin on GSH efflux, although inhibiting the sinusoidal component in the perfused liver, might also influence the "canalicular" component in the isolated cells. Our studies cannot resolve this uncertainty, although we can be certain that a significant component of the inhibitory effect of UCB is due to its action on the sinusoidal component.

In our efforts to establish the sidedness of inhibition, we set out to delineate whether UCB had to be taken up, before it could exert an inhibitory effect on the GSH efflux. The unequivocal demonstration of the inhibitory effect of bilirubin on GSH efflux being from inside the cell was made by taking advantage of an observation reported by Wolkoff et al. (16). Their work showed that the hepatic uptake of organic anions, including bilirubin, is dependent on the presence of extracellular $Cl^-$ and drops to zero values when $Cl^{-1}$ is absent in the media incubating hepatocytes or perfusing livers. Using $Cl^{-1}$-free buffer, made by replacing NaCl and KCl with equimolar sucrose in the Krebs buffer, we were able to show the total absence of the effect of UCB on GSH efflux from isolated hepatocytes and perfused livers. Not only could we show that uptake was necessary for inhibition but reversibility of the inhibitory effect required complete removal of the cellular bilirubin.

Another complication was the fact that UCB taken up by hepatocytes was rapidly conjugated to BMG and BDG. Thus, we could not be sure which molecular form was responsible for the inhibition of GSH efflux from inside the cell. The use of UCB and homozygote Gunn rat cells, that are incapable of producing BMG and BDG, unequivocally proved that the unconjugated form of bilirubin can inhibit the GSH efflux. On the other hand, when purified BMG and BDG were used, they too were taken up and inhibited GSH efflux from isolated Sprague-Dawley, heterozygote and homozygote Gunn rat hepatocytes. Thus, it appears that UCB, BMG, and BDG are each capable of inhibiting the efflux of GSH from hepatocytes. The similar potency of their effects, i.e., the degree of inhibition in relation to the extracellular concentrations of inhibitor used, precludes explaining the effect of BMG (and BDG) as due to deconjugation of the diglucuronide form to UCB (19). In our experiments, the inhibitory effect of the conjugated forms of bilirubin took place in the absence of detectable cellular levels of UCB. Thus, it seems doubtful that sufficient UCB could have been generated to explain the potency of the effects we observed with the conjugated forms of bilirubin.

Our kinetic data cannot be readily modeled to distinguish the exact nature of the inhibition and/or the accurate value of the kinetic parameters of inhibition, such as $K_i$. This is because we are not able to identify and measure the size of the intracellular pool(s) of the inhibitor that exerts an effect on the GSH efflux. With a simplifying assumption, it is possible to fit a purely competitive kinetic model to the data representing the % inhibition of GSH efflux vs. extracellular UCB or BMG concentration. The simplifying assumption is that, in the ranges of $K_m$ of inhibitor uptake, at which we have been working (20, 21), there might be a not-too-far from linear and one-to-one, dose-dependent relationship between the ECF and cellular concentrations of the inhibitor(s). Then, the Hill model that we have used to fit the kinetics of the uninhibited GSH efflux (3) can be expanded with a purely competitive inhibitory term, as presented before (14), to fit our present data for UCB and BMG incubations. The outcome of such fits, using the SAAM program (22), are shown by the curves in Figs. 4 and 5. Interestingly, the kinetic data from cells with elevated GSH can be simultaneously and consistently fitted along with that from the cells with normal GSH, using a single SAAM run (using the interrupt feature). Thus, with fairly stringent criteria for fitting the data with no more than two adjustable parameters, i.e., $K_i$ and $n$ (number of inhibitor sites) (14), good fits are obtained. The $K_i$ values extracted by our fitting exercises were $1.1\pm0.10$ and $0.54\pm0.13$ $\mu$M (mean$\pm$SD) for ECF UCB and BMG data, respectively. However, these ought to be regarded as "apparent" or fictitious $K_i$ values only, and the magnitude of the true $K_i$ values could be quite different from these. In both UCB and BMG experiments, the value of $n$, the number of cooperative inhibitor sites per carrier unit, did not exceed 1, implying the involvement of only a single inhibitor site.

In conclusion, we have shown that organic anions, but not bile acids, when added to the perfusion or incubation media, can inhibit the sinusoidal efflux of GSH from the perfused liver and isolated hepatocytes. Bilirubin at circulating plasma levels is capable of inhibiting the hepatic GSH efflux. All forms of bilirubin, i.e., UCB, BMG, and BDG are able to inhibit the efflux, and the inhibitory effect is exerted from inside the hepatocyte. These findings are not in conflict and, in fact, are consistent with the present knowledge and our working hypothesis of hepatic GSH efflux. Thus, organic anions enter the hepatocyte via a carrier(s), which is (are) distinct from the GSH carrier, but, once in the cell, compete with and inhibit the GSH carrier. On the other hand, the cellular GSH level has no effect on the uptake of bilirubin, but the apparent competitive inhibition by bilirubin of GSH efflux can be partially overcome by raising the cellular GSH concentration. It remains to be determined whether the efflux of organic anions is actually mediated by the GSH carrier.

Finally, our findings with respect to the effect of bilirubin on hepatic sinusoidal GSH efflux might have pathophysiological and clinical significance. Since the liver contributes > 90% of plasma GSH (2), during pathological states of hyperbilirubinemia, such as jaundice, it is possible that the efflux of he-

patic GSH to the blood might be inhibited to the extent that can cause a drop in the circulating GSH. This outcome could have deleterious effects on the whole organism's capacity for defense against xenobiotics and radical oxygen products, and, thus, give rise to further complications. On the other hand, the body may compensate by elevated hepatic GSH levels to overcome the inhibition and maintain normal levels of hepatic sinusoidal GSH efflux. These questions remain to be explored.

## Acknowledgments

We gratefully acknowledge the gift of Gunn rats by Dr. Anthony McDonagh of University of California, San Francisco, Liver Center. We also gratefully acknowledge the technical assistance of Michael Huott and Mei-Hui Hsu and the secretarial assistance of Ms. Anita Starlight.

This work was supported by National Institutes of Health grant DK-30312 and Veterans Administration Medical Research Funds. Dr. Fernandez-Checa is a recipient of fellowship awards from the American Liver Foundation and Smith-Kline-Beckman. Computing assistance was provided by intramural funds from the Department of Medicine, UCLA, for computations on the IBM 3033 at the Office of Academic Computing, UCLA.

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