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Morphine-Morphine Glucuronide Pools In the Rat Liver: Effects of Triton X-100[1]

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ABSTRACT

The purpose was to determine the effect on the morphine-morphine glucuronide systems of Triton X-100 instilled into the area of the hepatic canalicular membrane by segmented retrograde intrabiliary injection (40 μl of 0.4% Triton + 31 μl of saline) in the isolated *in situ* perfused livers of male Sprague-Dawley rats. In all experiments, [^{14}C]morphine was given by segmented retrograde intrabiliary injection (40 μl of [^{14}C]morphine + 110 μl of saline). The control single pass perfusate contained 15.8% [^{14}C]morphine glucuronide (MG) and 6.2% [^{14}C]morphine. With Triton, the major changes observed were an unusual plateau-like pattern of egress of the [^{14}C]MG into the perfusate and a profound decrease in the [^{14}C]MG excretion into bile. In controls, 45 mg of unlabeled morphine sulfate intraportally 5 min before the [^{14}C]morphine reduced the perfusate [^{14}C]MG and increased [^{14}C]morphine as expected by isotope dilution. Also, [^{14}C]MG recovery in bile was accordingly decreased (intact pentobarbital-anesthetized rat); however, the [^{14}C]morphine in bile did not change even though intracellular availability of [^{14}C]morphine must have been increased. In pulse chase experiments, [^{14}C] morphine was followed in 2 min with 45 mg of morphine sulfate intraportally. The perfusate [^{14}C]MG declined and [^{14}C]morphine increased. However, on the canalicular side (intact pentobarbital-anesthetized rat) no isotope dilution was evident: [^{14}C]MG and [^{14}C]morphine content in bile was similar to controls given no morphine sulfate. Triton treatment decreased the amount of [^{14}C] morphine which could be chased into the perfusate. Several different pools of morpine and MG exist in the liver; the canalicular and sinusoidal sites respond very differently to manipulation of these pools.

Much is known about the requirements of such factors as molecular weight, polarity and lipid solubility necessary for uptake, biotransformation and ultimate excretion of various compounds into bile (Smith, 1973). However, certain anomalous situations exist in which generally held concepts of hepatobiliary function do not seemingly apply such as with some compounds that are conjugated with UDP-glucuronic acid before excretion into bile. In phenobarbital-induced rats, the biliary excretion of MG was decreased despite an increase in its formation within the liver and an attendant choleresis (Roerig *et al.*, 1974; Peterson and Fujimoto, 1973). *Trans*-stilbene oxide treatment induced glucuronyl transferase and yet decreased the biliary excretion of MG and phenolphthalein glucuronide (Fuhrman-Lane and Fujimoto, 1982a,b). They suggested that compartmentalization of separate functions (conjugation and biliary excretion) might explain the effect of *trans*-stilbene oxide. An apparently analogous phenomenon has been observed with inducing agents such as chlordecone and Mirex (Mehendale, 1979) which decrease the biliary excretion of imipramine (as the glucuronide) in bile.

A group of investigators (Takada *et al.*, 1976; Nakae *et al.*, 1977a,b) used Triton X-100 injected retrogradely into the biliary tree of the rat to obtain a localized effect on the canalicular membrane of the hepatocyte. They showed that Triton treatment inhibited the transport of bromphenol blue into bile and released a canalicular membrane protein into bile which would bind bromphenol blue (Takada *et al.*, 1976). It is of interest that Triton X-100 is also used to activate glucuronyl transferase *in vitro* (Dutton, 1980; Denk, 1979). Inasmuch as our laboratory has developed a method of its own whereby various agents can be administered into the biliary tree of the rat, we wondered whether we could use Triton X-100 treatment to obtain localized effects on the canalicular membrane to further substantiate the existence of compartmentalization of conjugation of morphine and transport of MG. We also wanted to see if activation of glucuronyl transferase might be obtained by Triton treatment *in vivo*. If activation could be obtained *in vivo*, the results would indicate that the glucuronyl transferase in the endoplasmic reticulum is affected by the same membrane perturbation

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ABBREVIATIONS: MG, morphine glucuronide; SRII, segmented retrograde intrabiliary injection.

as the glucuronyl transferase in the microsomal preparation. While this study was in progress the interesting observation was made by Lasker *et al.* (1982) that flavone administration in the rat activates the hydroxylation of zoxazolamine, an enzymatic reaction carried out *in vitro*, by the microsomal mixed-function oxidase system. The major portion of the present study evolved into demonstrating the effect of Triton X-100 to disrupt the biliary excretion of MG and at the same time alter the transfer of MG from the liver into blood. Most importantly, we were able to show that morphine and MG reside in pools which can be manipulated experimentally in different ways. Furthermore, these pools could be shown to possess different orientations toward the sinusoidal and canalicular sides of the hepatocytes.

Methods

Animal preparation. Male Sprague-Dawley rats (ARS, Sprague-Dawley, Madison, WI), weighing 300 to 375 g, were anesthetized with pentobarbital sodium (45 mg/kg i.p.). After laparotomy, a femoral vein and the common bile duct were cannulated with Clay-Adams PE-50 and PE-20 polyethylene tubing, respectively. The proximal end of the bile duct cannula (10 cm in length with a 27-gauge needle at the distal end) was positioned just below the bifurcation of the common bile duct near the liver hilus. After surgery, a thermistor probe was inserted into the rectum and the temperature was measured with a Tele-Thermometer (Yellow Springs Instrument Company, Yellow Springs, OH). The animals were then placed near incandescent lamps to maintain body temperature at 37.5 ± 0.5°C.

Isolated *in situ* perfused rat liver preparation. The protocol utilized for studies performed in the isolated *in situ* perfused rat liver involved anesthesia and bile duct cannulation as described previously. Two hundred units of heparin sodium were injected into the abdominal aorta. The isolated *in situ* rat liver perfusion was prepared as described by Hems *et al.* (1966) and used by Fuhrman-Lane and Fujimoto (1982b). The portal vein was cannulated with a 16-gauge catheter. The inferior vena cava was cannulated by inserting a sharpened segment of a PE-205 polyethylene tubing through the right atrium. The hypoxic period did not exceed 2 min. The perfusion medium (100 ml) consisted of: washed (3 times) newly out-dated human erythrocytes (20 ml) to give a final hemoglobin concentration of 2.5 g; 2.5 g of bovine serum albumin and sufficient bicarbonate buffer (Krebs and Henseleit (1932), gassed with 95% O_2-5% CO_2, to bring the total volume to 100 ml. The pH of the perfusate was adjusted to 7.4 and the hematocrit measured. The perfusate was stored overnight at 4°C and then circulated and gassed in the humidified, heated perfusion cabinet. Livers were perfused *in situ* for a 30-min equilibration period. A thermistor probe was placed between the lobes of the liver and temperature maintained at 37.5 ± 0.5°C. Inflow hydrostatic pressure was established at about 16 cm of H_2O and the mean perfusion rate was 12 ml/min.

SRII procedure. The SRII procedure was performed for both of the above preparations as previously described (Olson and Fujimoto, 1980; Fuhrman-Lane and Fujimoto, 1982b). Briefly, in the SRII, an initial 40-μl "segment" of solution containing a given radioactive marker compound (0.2–0.5 μCi) was delivered into the bile duct cannula of the rat and "washed in" with 110 μl of 0.9% saline. The SRIIs were administered by an infusion pump (Harvard Apparatus Company, Dover, MA) at a rate of 2.3 μl/sec. Within 5 sec after the SRII injection, bile flow was resumed by detaching the injection apparatus from the bile duct cannula. Bile drops No. 1 to 10 and even-numbered drops No. 12 to 40 were serially collected in separate liquid scintillation vials. The total radioactivity recovered in bile drops 1 to 40 was then estimated and expressed as a percentage of the total dose of radioactivity administered by SRII. In the *in situ* liver preparation, simultaneously with the beginning of the administration of the marker compound, the venous outflow perfusate was collected in single pass fashion according to the method of Imamura and Fujimoto (1980a). The recycling perfusate from the interior vena cava was diverted at the reservoir and collected in 12-sec fractions.

When Triton X-100 was retrogradely administered into the biliary tree, 40 μl of buffered Tris-HCl-0.4% Triton solution (pH 7.4) + 31 μl of 0.9% NaCl was injected at a rate of 2.3 μl/sec. At the end of the injection, the segment was detached and bile flow resumed immediately. Controls received 71 μl of buffered 0.9% NaCl. Five minutes were allowed to elapse, bile flow was re-established and then marker compounds were administered.

Intraportal administration of [^{14}C]morphine was performed in one experiment with the isolated *in situ* liver preparation. In another series of experiments involving both the isolated liver and intact animal preparations, 45 mg of unlabeled morphine sulfate was injected into the portal vein over a period of about 1.5 min. Five minutes were allowed to elapse and then [^{14}C]morphine was administered by SRII. Another protocol with the same two preparations involved the initial SRII of [^{14}C]morphine, followed by intraportal injection of 45 mg of unlabeled morphine sulfate at 2 min into the liver perfusion.

Analysis of radioactive morphine and MG. Procedures for analysis of bile and venous outflow perfusate samples were as previously described (Imamura and Fujimoto, 1980a) and similar in principle to that to be given in detail for the liver. In isolated perfused liver experiments, after SRII administration of [^{14}C]morphine, the liver was removed at the end of the collection of 40 drops of bile and homogenized with 80 ml of distilled water. The homogenates were centrifuged after the addition of 0.5 volume of 30% (w/v) trichloroacetic acid. A 100-μl sample of supernatant was assayed for the radioactivity represented by total morphine in a liquid scintillation mixture (ACS, Amersham/Searle, Arlington Heights, IL). Free radioactive morphine was determined by adjusting the pH to 8.5 of 1 ml of liver homogenate with 6 M NaOH and then extracting the mixture with 2 ml of water-saturated, ethyl acetate-butanol (7/3 v/v). A 500-μl sample of the organic phase was then assayed for radioactivity in a Triton-toluene liquid scintillation solution. These counts were then corrected for the extraction efficiency of 76.5% (±5.4) for [^{14}C]morphine added to and recovered from the liver homogenate. The content of radioactive MG was then obtained as the difference between the total radioactivity and the free [^{14}C]morphine.

Statistical analysis. When statistical evaluation was necessary, results were compared by the Student's unpaired *t* test and $P \leq .05$ was taken as indicating a significant difference. In addition, the multiple *t* test of Dunnett (1964) was used to compare the effect of "preload" and "pulse-chase" unlabeled morphine on the content of [^{14}C]morphine and [^{14}C]MG in the control and Triton-treated *in situ* perfused livers.

Drugs and chemicals. Sources of drugs and chemicals were as follows: Triton X-100 was obtained from Calbiochem (San Diego, CA); pentobarbital sodium was obtained from Henry Schein, Inc. (Port Washington, NY); morphine sulfate was obtained from the Malinckrockdt Chemical Works (St. Louis, MO); heparin sodium and bovine albumin, Fraction V (Lot No. 40F-0266) were from Sigma Chemical Company (St. Louis, MO); Protosol and Biofluor were from New England Nuclear (Boston, MA). Outdated packed human erythrocytes (used within 5 days of their expiration date) were obtained from the Milwaukee Blood Center (Milwaukee, WI). [N-$^{14}CH_3$]morphine hydrochloride (58 μCi/mmol) was from Amersham/Searle and [N-$^{14}CH_3$]morphine-3-glucuronide (0.45 μCi/mg) was biosynthesized (Fujimoto, 1969). Radioactive purity of the compounds was as determined earlier (Fuhrman-Lane and Fujimoto, 1982a). All compounds were administered in 0.9% saline. All other chemicals were of reagent grade.

Results

Figure 1 (panel A) demonstrates the movement of ^{14}C into the perfusate after intraportal administration of [^{14}C]morphine in the isolated *in situ* perfused rat liver preparation. In the control, the recovery of total ^{14}C counts was 33% of the administered dose, of which [^{14}C]morphine accounted for 19.6% and [^{14}C]MG for 13.4%. The initial large peak concentration of ^{14}C

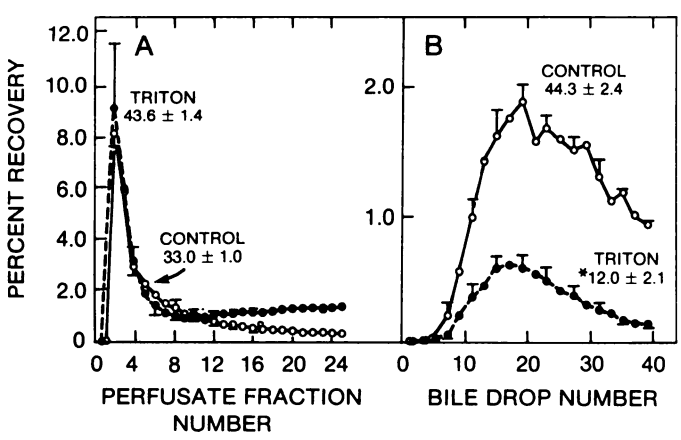

Fig. 1. Effect of 0.4% Triton X-100 treatment on the concentration of ^{14}C-radioactivity in the venous outflow (A) and bile (B) of the perfused liver after intraportal injection of [^{14}C]morphine. The Triton was administered as a 40-μl segment into the cannulated bile duct, followed by 31 μl of saline at a rate of 2.3 μl/sec. Controls received 71 μl of saline. Five minutes elapsed before injection of [^{14}C]morphine. The points on each curve represent the percent recovery (mean ± S.E.) of the administered dose in each perfusate fraction and bile drop collected after the injection in four control (O) *vs.* four Triton-treated (●) rats. * Differs significantly from control, $P < .05$.

was observed at perfusate fraction number 2 to 3, which probably represented in large part that portion of the administered ^{14}C which was not extracted by the liver during its first pass through the organ. After retrograde intrabiliary treatment with 0.4% Triton X-100, the recovery of total ^{14}C in the perfusate after the intraportal administration of [^{14}C]morphine was approximately 44% of the administered dose. Free [^{14}C]morphine accounted for 25% of the total recovery, whereas [^{14}C]MG at 18.5% represented the remainder. Although the Triton-treated group displayed an early peak of radioactivity in the perfusate, the later perfusate fractions were found to contain higher concentrations of radioactivity than corresponding control samples. This increase in perfusate concentration of radioactivity was observed from samples 16 to 25. However, the effect of Triton X-100 treatment was not particularly obvious.

Figure 1 (panel B) demonstrates the accompanying biliary excretion of ^{14}C after intraportal administration of [^{14}C]morphine in the isolated perfused liver. For the control, 44% of the administered ^{14}C was excreted in 40 drops of bile, of which 2.2% was free morphine and 42% was MG. After Triton treatment, a marked difference in the biliary recovery of total ^{14}C occurred. Biliary recovery of free morphine fell to 0.4%, but the largest decrease was observed in the content of [^{14}C]MG which decreased to 12% from the control value of 42%. The overall recovery of ^{14}C for bile plus perfusate was 77 and 56% in control and Triton treated preparations, respectively.

Preliminary experiments demonstrated the pattern of movement of ^{14}C into the perfusate after SRII of [^{14}C]morphine in the perfused liver preparation. In the control ($N = 5$), 20.9 ± 1.0% of the total administered ^{14}C was present in the 25 samples of perfusate, of which 15% was represented by [^{14}C]MG and 6% by morphine. The egress pattern of the ^{14}C-derived radioactivity from liver into perfusate displayed a peak blood concentration at fraction 6 and the concentrations then decreased in apparent first-order fashion.

In perfused livers which had been treated with 0.4% Triton X-100 ($N = 5$), the total ^{14}C present in perfusate (23.1 ± 2.4%) was not significantly different from that in the control, with [^{14}C]morphine comprising about 8% and [^{14}C]MG about 16%. However, the concentration profile of the ^{14}C in the perfusate was vastly different from that observed in the control. Whereas the peak concentration was present at approximately fraction 4, the movement of ^{14}C thereafter from liver into perfusate assumed a plateau-like profile out to the end of the sample collection. Therefore, whereas the total radioactivity recovered, as well as that of the individual constituent species, were not different from one another between the groups, the kinetic profile of egress of ^{14}C was drastically altered.

The recovery and profile of ^{14}C-recovery in bile after SRII of [^{14}C]morphine in the same set of experiments were changed. In the control, 47.2 ± 2.7% of the total administered dose was excreted into bile. Free [^{14}C]morphine constituted 6% and [^{14}C]MG accounted for about 41%. A peak concentration of ^{14}C-radioactivity was observed at approximately bile drop 27. After the treatment with Triton, a decrease to 22.6 ± 0.7% in the biliary recovery of total ^{14}C occurred. Whereas the content of free morphine in bile rose slightly (from 6 to 12%), that of MG decreased by 30%. The ^{14}C-concentration profile was shifted to the left, with a peak concentration observed at bile drop 5.

The experiments employing the SRII of [^{14}C]morphine were repeated in order to obtain a more detailed profile of the concentrations of [^{14}C]morphine and [^{14}C]MG in the individual perfusate and bile samples. In the control, figure 2A, free [^{14}C]morphine recovery in the perfusate was about 6% and peaked at fraction 6. [^{14}C]MG accounted for 16% of the total ^{14}C administered, peaked at about fraction 9 and then declined in apparent first-order fashion. In figure 2 panel B, after treatment with Triton X-100, the recovery of [^{14}C]morphine in the perfusate was comparable with that in the control in quantity

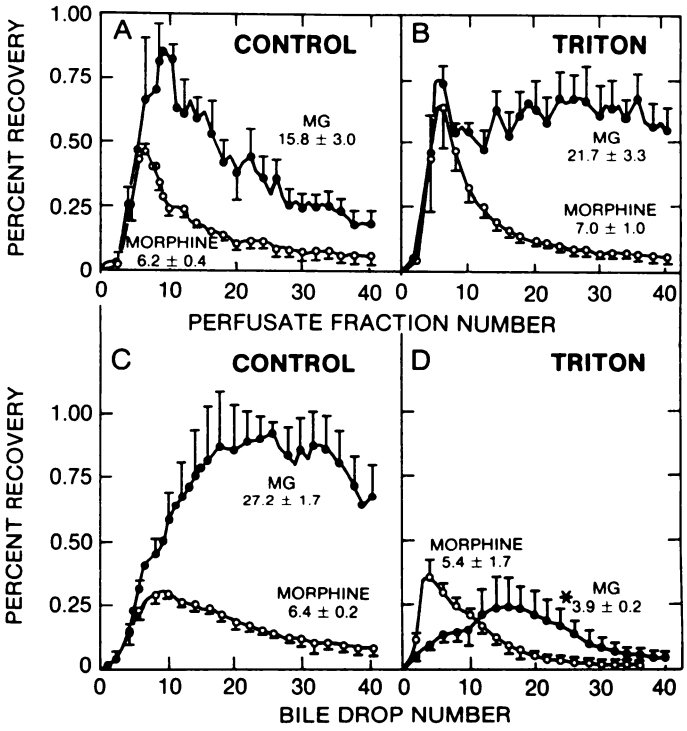

Fig. 2. Effect of Triton X-100 administration on the recovery of [^{14}C]morphine and [^{14}C]MG in the venous outflow (A and B) and bile (C and D) of the perfused rat liver after SRII of [^{14}C]morphine. The data are plotted in the previous format, ($N = 4$). * Differs significantly from control, $P < .05$.

and pattern of egress into the perfusate. Whereas the overall recovery of [^{14}C]MG appeared to be unaffected by Triton treatment (16 *vs.* 22%), the profile of egress of [^{14}C]MG was drastically changed. After an initial peak in perfusate fraction 6, the remainder of the fractions displayed a plateau-like pattern of egress of [^{14}C]MG. Thus, the difference seen between control and Triton treatment groups was largely by an effect on [^{14}C]MG.

The corresponding results for the analysis of radioactivity in the bile of the control and Triton-treated groups are given in figure 2, C and D. The recovery of [^{14}C]morphine in control (6.4%) and Triton-treated (5.4%) groups was the same but the curve for the latter group was shifted to the left. MG recoveries of control (27.2%) and Triton-treated (3.9%) groups were very different.

Because the results in figure 2B for the Triton treatment indicated that the plateau in [^{14}C]MG egress into the perfusate appeared to persist well beyond the last fraction collected, number 40, a separate experiment was performed in which the perfusate collection was carried out further to 70 fractions. The result seen in figure 3 indicated that the plateau persisted even beyond perfusate fraction 70. A control for collection of 70 perfusate fractions was not run because the control for the collection of 40 fractions (figure 2A) indicated that beyond fraction 40 only small amounts of ^{14}C would be recovered.

Inasmuch as the predominant effect of the Triton treatment was to affect the passage of the MG (formed from morphine) from the liver into the perfusate and bile, we studied the transfer of the MG itself. The results for the control, in which [^{14}C]MG was given by SRII, demonstrated that the radioactivity appeared rapidly in the perfusate. The peak was in fraction number 3 and by number 8 most of the egress was completed. This rapidity of fall of radioactivity contrasted with the results when [^{14}C]morphine was given (fig. 2), where the radioactivity reached a peak later and came into the perfusate over a more prolonged period of time. After Triton X-100 treatment in the present experiment, the concentration peak in the perfusate was depressed compared with the control. However, the total ^{14}C-recovery was not different (control: 61.9 ± 3.9 *vs.* Triton: 61.2% ± 3.4%, $N = 4$). It seemed that the exogenously administered MG moved so rapidly through the liver to enter the perfusate that, even though the Triton treatment might have had an effect, the effect did not appear to be protracted under the circumstances. For bile collected in the intact animal after SRII of [^{14}C]MG, the recovery of [^{14}C]MG was decreased from a control value of 33.2 ± 3.2 to 7.3 ± 1.3% in the Triton-treated group.

A different approach was taken in the next series of experiments to attempt to further elucidate the nature of the defect caused by Triton treatment. In the first set of experiments, the perfused liver system was given a load of 45 mg of morphine sulfate intraportally. Five minutes later, the usual SRII administration of [^{14}C]morphine was performed. Figure 4 (panel A) for the control group shows that the major portion of the ^{14}C-derived radioactivity in the perfusate was now present as free morphine (29%). Note that the free morphine reached a peak rapidly and thereafter declined in an apparent first-order fashion. In contrast to figure 2, the proportion of free morphine to MG was approximately reversed. The low recovery of MG of 8.7% in figure 4A indicated that the earlier loading of the liver with unlabeled morphine was effective in reducing the amount of [^{14}C]MG in the perfusate. After Triton treatment (fig. 4B), the amounts and profiles of free morphine and conjugate found in the perfusate were similar to those in the control. The main conclusion from this experiment was that preloading the liver with morphine was effective in causing much more free [^{14}C]morphine to appear in the perfusate. Also, the amount of glucuronide was drastically reduced. However, Triton treatment did not have any easily discernible effect under this condition.

Figure 5 demonstrates the effect of administering the [^{14}C]morphine by SRII first and then 2 min later administering the 45 mg of unlabeled morphine sulfate intraportally (a pulse-chase experiment). In the control (fig. 5A), both free [^{14}C]morphine and [^{14}C]MG began to enter the perfusate before the unlabeled morphine bolus entered the liver. At this point, the administration of unlabeled morphine rapidly shut down further appearance of the conjugate in the perfusate and "chased" free [^{14}C]morphine out of the liver. Note the rapid rise in the [^{14}C]morphine concentration and its rapid decline in the perfusate. In the Triton-treated group (fig. 5B), administration of the unlabeled morphine decreased the [^{14}C]MG concentration as in the control. The recovery of 3.2% was not different from the control value of 4.8%. Examining the free [^{14}C]morphine concentration after the point where the unlabeled morphine was administered, the peak concentration attained was only

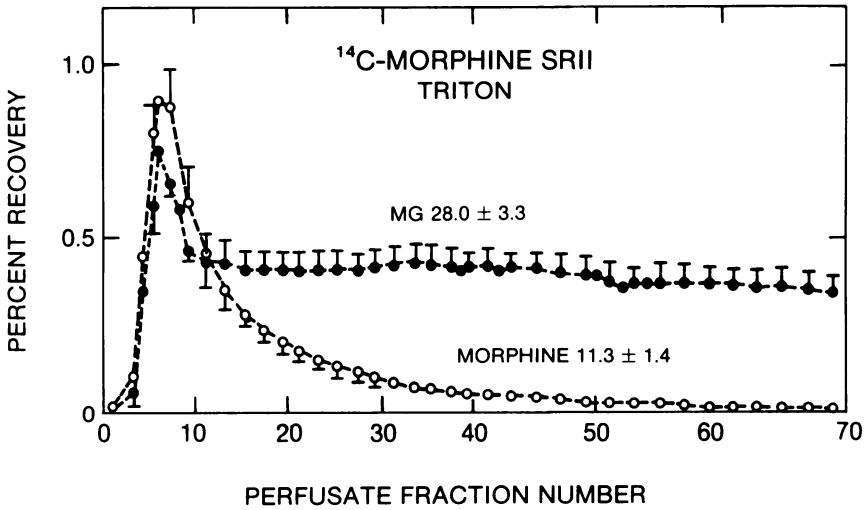

Fig. 3. Effect of Triton treatment on the recovery of [^{14}C]morphine and [^{14}C]MG on the venous outflow of the perfused rat liver after SRII of [^{14}C]morphine. The perfusate was collected out to 70 fractions; in other respects this experiment was similar to that in figure 2B. The data are plotted in the previous format ($N = 6$). Both MG and morphine recoveries differ significantly from control, figure 2A, $P < .05$.

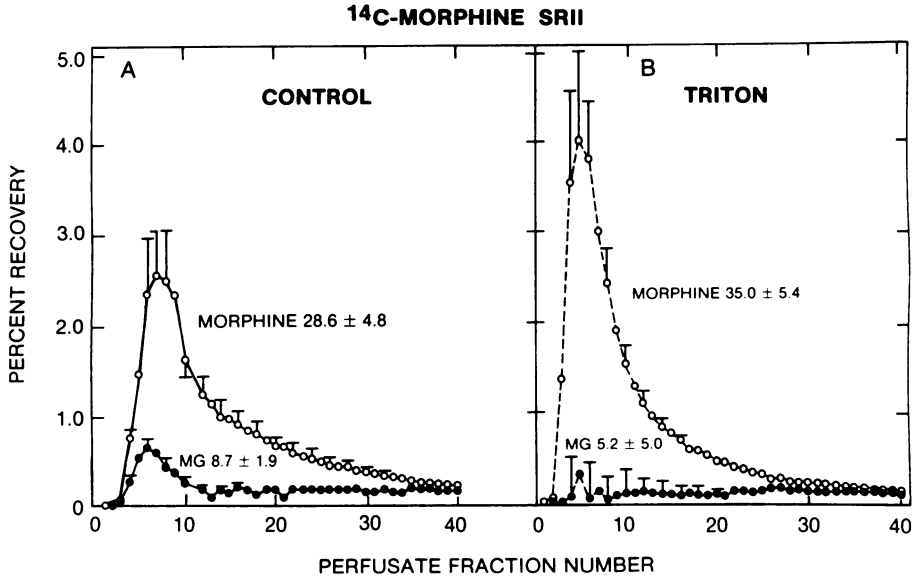

Fig. 4. Effect on the venous outflow recoveries of [^{14}C]morphine and [^{14}C]MG in control (A) and Triton-treated (B) perfused livers given a large dose of morphine intraportally before the SRII of [^{14}C]morphine. The Triton was administered 5 min before the intraportal injection of 45 mg of cold morphine sulfate. Five minutes later, [^{14}C] morphine was administered by SRII. The data are plotted in the previous format (N = 4).

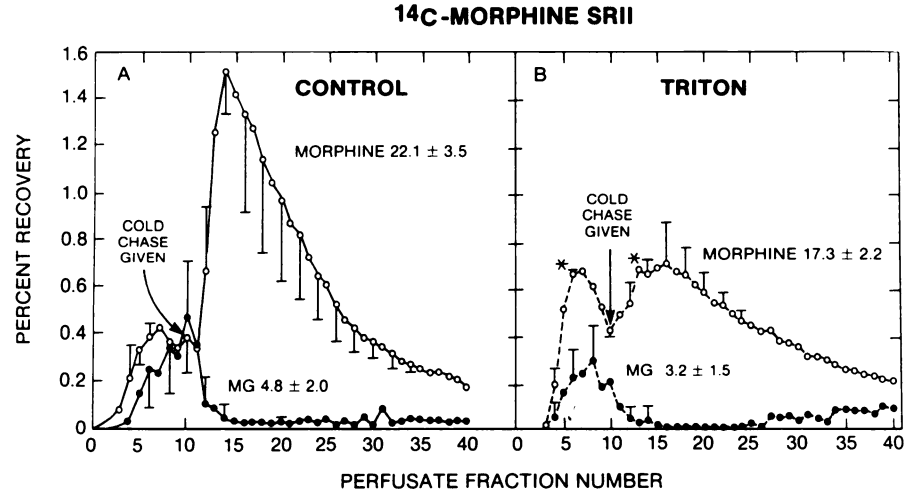

Fig. 5. Effect on the venous outflow recoveries of [^{14}C]morphine and [^{14}C]MG in control (A) and Triton-treated (B) perfused livers given the SRII of [^{14}C]morphine followed by a large dose of morphine intraportally. Triton was administered 5 min before the SRII of [^{14}C]morphine. Two minutes after the start of the SRII, 45 mg of morphine sulfate was intraportally injected. The data are plotted in the previous format (N = 4). * Differs significantly from control, $P < .05$.

about one-half as great as in the control. Thereafter, the [^{14}C] morphine concentration declined slowly. However, when we plotted the latter portions of this and the control curve, we found that the first-order rate constant was only slightly reduced. Thus, the main effect could be attributed to the reduced peak concentration; namely, the Triton treatment somehow decreased the amount of free morphine able to enter the perfusate, but that which was released entered with a similar first-order rate constant as in the control.

In these experiments bile samples were not collected because of the extra attention required of the investigator to carry out the experiment. Thus, a separate set of *in vivo*, experiments was carried out to assess the effects on biliary excretion. Figure 6 (panel A) presents results for the control group given a 45-mg intraportal injection of morphine sulfate, followed in 5 min by the SRII of [^{14}C]morphine. It is seen that the recovery of 4.7% for the [^{14}C]morphine was similar to the 6.4% obtained in figure 2C. However, the curve for [^{14}C]MG did not rise as sharply here as in figure 2C and the recovery changed (27 *vs.* 9%). Thus, in the control situation, giving the unlabeled morphine load decreased the [^{14}C]MG excretion but not the free [^{14}C]morphine component. The experiment carried out after Triton X-100 treatment (6B) again showed that the presence of unlabeled morphine made no difference in the content of the free [^{14}C]morphine recovery curve in bile compared with the result in figure 6A. Because the Triton treatment had drastically reduced the amount of [^{14}C]MG, as seen in figure 2D, it was not surprising that administration of the unlabeled morphine in the experiment in figure 6B had no further discernible effect on the subsequent biliary [^{14}C]MG recovery compared with figure 6A. However, the shape of the biliary excretion curve may have been slightly different. The conclusions from this set of experiments were that the unlabeled morpine loading decreased the amount of [^{14}C]MG in bile; a corresponding rise in [^{14}C]morphine did not occur in bile even though more [^{14}C] morphine should have been available from the liver. The increased availability of [^{14}C]morphine is inferred from the increase in perfusate concentration of free [^{14}C]morphine seen in the earlier experiments (fig. 4, A and B). Under the conditions of this experiment, Triton treated-animals and controls were not different.

In another experiment, the *in vivo* biliary excretion of [^{14}C] MG and [^{14}C]morphine after the SRII of [^{14}C]morphine in the pulse-chase format was examined. Figure 7 (panel A) illustrates that in the control the large concentration of unlabeled morphine entering the liver 2 min after the SRII of [^{14}C]morphine

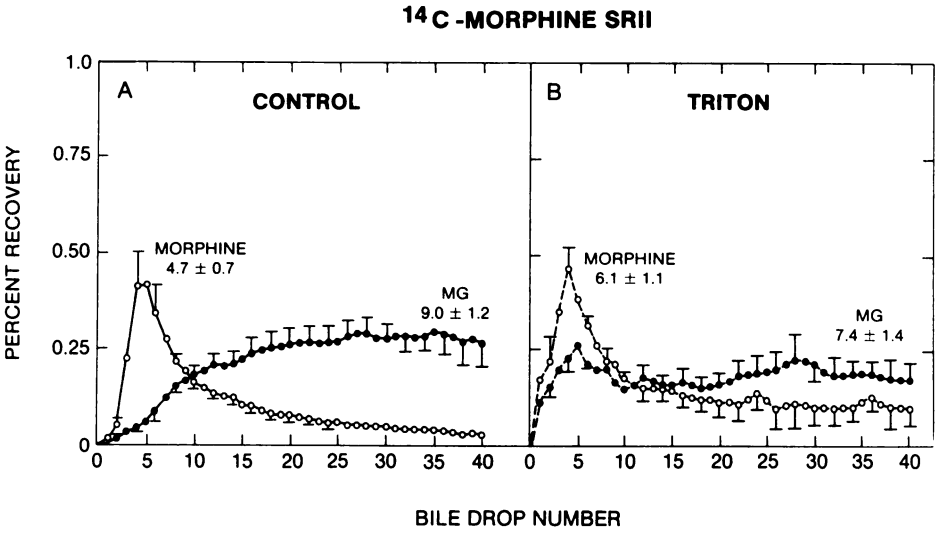

Fig. 6. Effect of the biliary recoveries of [^{14}C] morphine and [^{14}C]MG in control (A) and Triton-treated (B) pentobarbital-anesthetized rats given a large dose of morphine intraportally before the SRII of [^{14}C]morphine. The protocol utilized was that employed in figure 4: Triton (SRII) 5 min before 45 mg of morphine sulfate (intraportally), 5 min later the SRII of [^{14}C]morphine. The data are plotted in the previous format (N = 4).

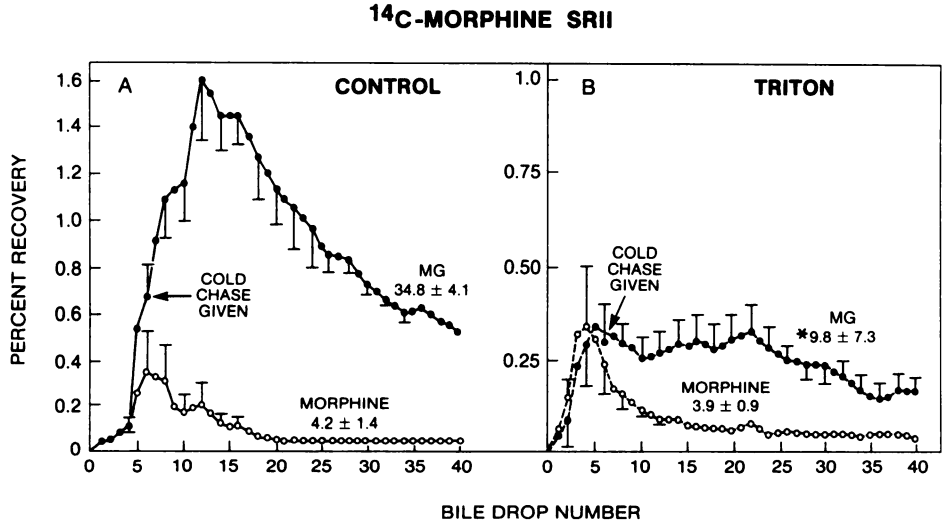

Fig. 7. Effect on the biliary recoveries of [^{14}C] morphine and [^{14}C]MG in control (A) and Triton-treated (B) pentobarbital-anesthetized rats given the SRII of [^{14}C]morphine followed by a large dose of morphine intraportally. The protocol utilized was that employed in figure 5: Triton (SRII) 5 min before SRII of [^{14}C] morphine, followed in 2 min by 45 mg of morphine sulfate (intraportally). The data are plotted in the previous format (N = 4). * Differs significantly from control, $P < .05$.

did not decrease the amount of [^{14}C]MG in bile (34.8%) compared with the previous control 27.2%, figure 2C. However, whereas the peak MG concentration in the control in figure 2C was present at a later biliary volume, the peak concentration in this experiment was at bile drop No. 12. The amount of [^{14}C] morphine recovered in bile was the same as that observed in figure 2C (6.4% vs. 4.2%).

Figure 7B demonstrates that in the Triton-treated group the biliary excretion of [^{14}C]morphine was similar to that in the control group (fig. 7A). Even though the unlabeled morphine chase increased [^{14}C]morphine in the perfusate, [^{14}C]morphine excretion into bile was not increased. The [^{14}C]MG recovery was decreased compared with the control (fig. 7A). However, it should be noted that because the experiment in figure 2D had shown a similar reduced recovery, it is likely that the present reduced recovery was probably not due to the effect of the unlabeled morphine chase.

One further parameter was measured: liver content of [^{14}C] morphine and [^{14}C]MG at the termination of the isolated perfused liver experiments. The first set of data in table 1 indicate the amount of [^{14}C]morphine and MG left in the perfused livers after SRII of [^{14}C]morphine at the end of the experiment depicted in figure 2. In the control situation, approximately 55% of the total administered ^{14}C was estimated to be in the liver. Of that amount, approximately 14% was [^{14}C] morphine and 42% [^{14}C]MG. After treatment with Triton, approximately 36% was [^{14}C]MG. Considering the recoveries in perfusate, bile and liver, in the control 111% of the administered ^{14}C was accounted for in the analyses; for the Triton-treated group, 88% was accounted for. This 12% difference in unaccounted ^{14}C cannot be explained. Such differences in accounting for the ^{14}C-balance also appeared to exist in some of the other experiments listed in table 1. Note the recoveries in table 1 for the Triton group in which 70 *vs.* 40 perfusate samples were collected. The livers from the latter experiment showed a decrease in the [^{14}C]morphine content (7.5 *vs.* 14.0%) and no change in the [^{14}C]MG content (33.5 *vs.* 36.2%). Thus, it appears that the increase in perfusate [^{14}C]MG recovery of about 7% observed in going from the 40 to 70 perfusate sample collection (in fig. 2 *vs.* fig. 3) coincides with the decrease in the [^{14}C]morphine content in the liver.

The data in table 1 for the unlabeled morphine preload experiment are consistent with the expectations from figures 4 and 6. No differences are observed between the liver recoveries

TABLE 1

Amount of [^{14}C]morphine and [^{14}C]MG (expressed as percent of the administered dose of [^{14}C]morphine, mean ± S.E.) in the isolated liver at the end of the various experiments

The experiments corresponding to the analysis shown here are designated in this table by the figure numbers.

	Control	Triton	Corresponding Exper.
	% of administered ^{14}C		
[^{14}C]Morphine	13.6 ± 2.5	14.0 ± 3.1[a]	Figure 2
[^{14}C]MG	41.7 ± 2.6[b]	36.2 ± 4.0[c]	
[^{14}C]Morphine		7.5 ± 0.1[a*]	Figure 3
[^{14}C]MG		33.5 ± 4.6	
[^{14}C]Morphine	18.9 ± 3.9	13.2 ± 2.7[c]	Figure 4
[^{14}C]MG	19.1 ± 3.0[b*]	20.6 ± 2.1[c*]	Morphine sulfate load
[^{14}C]Morphine	22.0 ± 5.4	35.6 ± 5.6[a*]	Figure 5
[^{14}C]MG	24.0 ± 4.5[b*]	7.5 ± 3.8[c*]	Morphine sulfate chase

[a*],[b*],[c*] Significantly different from [a], [b] and [c], respectively, $P < .05$ (Dunnett's test).

of ^{14}C-components in the control and Triton-treated groups. Preloading with unlabeled morphine had the effect in both control and Triton-treated groups of increasing [^{14}C]morphine and decreasing [^{14}C]MG recovery in the perfusate (fig. 4). The decrease in [^{14}C]MG in the perfusate was consistent with a decrease in liver content of the conjugate. However, the [^{14}C] morphine content was similar to that observed when the morphine preload was not performed.

The last set of data in table 1 is derived from the pulse-chase experiments. One finding is not consistent with expectations from figures 5 and 7. Namely, we expected that the reduced total recovery of ^{14}C produced by Triton treatment would be reflected by an increased total ^{14}C-recovery of the remaining balance in the liver. The total ^{14}C content in liver was 46% for the control group and 43% for the Triton-treated group. It is, however, very interesting that the relative content of [^{14}C] morphine to [^{14}C]MG in the control liver is about 1 to 1, whereas that for the Triton liver is about 1 to 1/4. This latter observation will be used to support our contention that the unlabeled morphine chase is somehow less effective in releasing [^{14}C]morphine from the liver of the Triton group than the control group.

Discussion

The predominant effect seen after Triton treatment was a reduction in the amount of [^{14}C]MG excreted into bile after [^{14}C]morphine administration. Such a result could have been produced by a decreased uptake of [^{14}C]morphine into the liver and thereby a decreased availability of [^{14}C]morphine for conjugation; absence of an increased loss of [^{14}C] morphine in the perfusate in the Triton-treated group militated against this possibility. Two other mechanisms, inhibition of MG formation and inhibition of its excretion into bile, were then considered.

After SRII administration of [^{14}C]morphine in Triton-treated animals, the concentration profile of the free [^{14}C] morphine in the perfusate remained about the same as in the control group (fig. 2), but that for [^{14}C]MG approximated an apparent zero-order process manifested as a plateau. This effect was discovered because the [^{14}C]morphine was given by SRII. When the [^{14}C]morphine was given intraportally, the presence of such an effect was not even suspected. The plateau was due to the MG component. On balance, the amount of [^{14}C]MG accounted for in the experiment in figure 2 for perfusate and bile in the Triton-treated group (21.7 + 3.9 = 25.6%) was less than in the control (15.8 ± 27.2 = 43%); these findings appear to indicate that Triton treatment inhibited the conjugation of morphine.

Certain results, however, cloud this interpretation. Data for the same experiment tabulated in table 1 showed that the ratio of [^{14}C]morphine to [^{14}C]MG in the liver was not changed by the Triton treatment. There was more MG than morphine, as in the control. We would expect the relative amount of MG to decrease if glucuronidation were inhibited. For instance, the same table shows that in the control liver preloaded with unlabeled morphine, the ratio of [^{14}C]morphine to [^{14}C]MG increased toward 1. The isotope dilution by the morphine decreased the formation of [^{14}C]MG. Inhibition of MG formation in rats pretreated by SKF 525A also leads to an increase in the ratio of morphine to MG in the liver (Liu and Wang, 1980) in spite of some trapping of MG in the liver (Imamura and Fujimoto, 1980c). In figures 2 and 3, the initial peak in [^{14}C]MG in the perfusate was just as high in the Triton group. If MG were inhibited, the height of this peak should have been reduced. *In vitro* work with microsomes prepared from Triton-treated and control livers (Franklin *et al.*, 1982) indicated that morphine glucoronidation was not inhibited. Thus, the plateau of MG egress into the perfusate was not produced by a simple inhibition of glucuronyl transferase.

It seemed unlikely that inhibition of canalicular transport of MG into bile would in itself lead to the plateau; even though movement of exogenously-administered [^{14}C]MG into the perfusate was decreased, no plateau was seen. Also dehydrocholate given i.v. inhibit the biliary excretion of intracellularly formed MG (Imamura and Fujimoto, 1980b), but no plateau is seen in the perfusate.

The results from the unlabeled morphine loading and pulse chase experiments suggested other alternatives; the mechanism controlling the egress of morphine from the canalicular side of the cell was not the same as for the sinusoidal side. In both control and Triton-treated groups (fig. 4), increased amounts of free [^{14}C]morphine entered the perfusate in the presence of the load of unlabeled morphine. Biliary excretion of [^{14}C] morphine (fig. 6) was unaffected even though there must have been increased amounts of [^{14}C]morphine available. The [^{14}C] morphine excreted in bile was resistant to manipulation, whereas that which appeared in the perfusate was more easily manipulated. Therefore, a definite orientation, a sidedness or compartmentalization occurs in the hepatocyte.

Glucuronidation of [^{14}C]morphine was inhibited by isotope dilution; loading the liver with unlabeled morphine produced a drastic decrease in the amount of [^{14}C]MG in the perfusate (fig. 4A) and bile (fig. 6A) of the control and in the perfusate of the Triton group (fig. 4A). It was possible to chase away the [^{14}C] morphine from the glucuronyl transferase. In figure 7, when [^{14}C]morphine was given first, followed 2 min later by unlabeled morphine chase, the concentration of [^{14}C]MG abruptly decreased in the perfusate. Because a corresponding rise in [^{14}C] morphine egress occurred, we concluded that [^{14}C]MG synthesis must have been turned off. In spite of this effect, there must be a pool oriented toward biliary excretion into which the morphine chase could not gain equal access. This chase was not effective in turning off the appearance of [^{14}C]MG (35%)

in bile. Either the [^{14}C]glucuronide had been formed before the cold morphine chase could affect the availability of the [^{14}C] morphine, or the chase was ineffective in displacing the [^{14}C] morphine. Thus, the most attractive suggestion would be that the MG within the cell exists in more than one homogenous pool. One pool turns over rapidly and is recognized by its contribution of MG to the blood and is sensitive to the unlabeled morphine chase; the other is recognized by its insensitivity to the unlabeled morphine chase and is oriented toward biliary excretion.

In figure 5, when the areas under the curve (recovery) for [^{14}C]morphine after the chase was given were compared, the recovery is less for the Triton-treated group than the control. Inasmuch as the unlabeled morphine chase was equally effective in decreasing the appearance of [^{14}C]MG in the perfusate, we feel that the decreased ability of the morphine to chase out [^{14}C]morphine was an important effect manifested by the Triton treatment.

Theoretically, there are two ways in which the effectiveness of the chase could be decreased by Triton treatment. Either the binding of [^{14}C]morphine to hepatic tissue was increased so that unlabeled morphine could not as effectively compete for binding or the pool of [^{14}C]morphine presumably in free solution was increased. Presumably, [^{14}C]morphine in solution cannot be displaced by adding morphine. In contrast, in the control both the pulse-chase and morphine preload experiments indicate that a large portion of the intrahepatically-sequestered [^{14}C]morphine was displaceable by unlabeled morphine. Thus, this morphine must have been present in a displaceable, tissue bound form. For our purposes most of this displaceable morphine can be assumed to come from relatively low-affinity binding sites. Alternatively, Triton treatment may increase high-affinity binding of morphine as some prolonged retention of morphine is normally seen in rats (Mullis *et al.*, 1979; Jones *et al.*, 1983). When accounting for ^{14}C is done for bile, perfusate and liver recoveries, 89 to 112% recoveries were found in the control experiments; the recovery in the Triton experiments ranged from 76 to 88%. The problem in not obtaining a 100% balance resides in analytical problems with the measurement of liver samples; such difficulties might be speculated to relate to the binding of the ^{14}C.

The major findings with Triton, the plateau in the [^{14}C]MG pattern for the perfusate (fig. 2B) may be explained in terms of pools of morphine glucuronide. In comparing figure 2, A and B, the initial peak in perfusate [^{14}C]MG concentration was not affected by the Triton treatment. This peak may correspond to the pool which is recognized by its ability to be disrupted by the unlabeled morphine chase. The other pool which was recognized by its insensitivity to the morphine chase and was oriented more to biliary excretion would be the pool affected by the Triton. Thus, biliary excretion of MG is inhibited and this glucuronide is directed into the perfusate for a protracted period (fig. 3); a phasic difference becomes apparent.

Compartmentalization in the morphine-MG system was suggested earlier. Fuhrman-Lane and Fujimoto (1982a,b) found that even though *trans*-stilbene oxide treatment of rats induced morphine glucuronyl transferase, the increased intrahepatic availability of MG was associated with a decrease in its biliary excretion. Similarly, the phenobarbital-induced increase in morphine glucuronidation has been associated with a decrease in biliary excretion of MG (Peterson and Fujimoto, 1973; Roerig *et al.*, 1974). Mehendale (1979) found that with certain other inducers biliary excretion of imipramine glucuronide was decreased. It may be that changes in pools or compartments might explain his findings (personal communication). Gessner and Hamada (1974) suggested that an intracellularly formed glucuronide was in a pool within the cell which was not readily accessible to the systemically circulating glucuronide.

In conclusion, we should emphasize that our findings support the concept of the existence of definite differences in control mechanisms for biliary excretion and sinusoidal excretion of metabolites formed within the liver. Experimental manipulation has made the presence of such mechanisms evident. The recognition of intracellular pools is of paramount importance in gaining a new perspective into the mechanism of biliary excretion.

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