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BILIARY EXCRETION AND ENTEROHEPATIC CIRCULATION OF PREGNANOLONE IN THE RAT*

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Abstract—Studies were made of the metabolism and biliary excretion of pregnanolone, a metabolite of progesterone exhibiting a variety of pharmacologic activities, in rats bearing venous and biliary catheters. Pregnanolone glucuronide appeared rapidly and in large amounts in the bile, with a concentration gradient in the order of 400 relative to serum. The relative concentration of various metabolites in the bile remained constant over a 5-day period. An enterohepatic cycle was established by finding peaks in the serum concentration of pregnanolone after intragastric administration of bile collected from pregnanolone-pretreated rats, containing pregnanolone and pregnanolone glucuronide. It appeared that hydroxylation of pregnanolone at C6 and 17 and reduction of C20 occurred during its passage through the intestine.

After metabolism by hepatic microsomal enzymes [1–6], many drugs and steroids are excreted via the bile. For some years we have studied the metabolism, distribution and excretion of 5β-pregnan-3α-ol-20-one (pregnanolone†), a naturally occurring steroid possessing a variety of pharmacologic properties [7,8]. This steroid is a metabolite of progesterone, and has been found in the urine in large amounts (about 10 mg/day) during pregnancy [9].

Our previous studies in rats [7,8] indicated that, despite rapid clearance from serum, only about 8 per cent of an i.p. dose appeared in the urine during 6 hr. Since large amounts were found in the intestine, the present study was designed to quantitate the rate of biliary excretion of pregnanolone, identify metabolites and to determine the presence of an enterohepatic circulation.

MATERIALS AND METHODS

Animals and surgical procedures. Sprague–Dawley male rats, 200–300 g, were obtained from Simonson Laboratories (White Bear Lake, Minn.). Two days or more before the experiment, they were anesthetized with pentobarbital (45 mg/kg) and a chronic intravenous cannula was placed in the jugular vein by the procedure of Davis and Campbell [10]. The cannula was filled with heparinized polyvinyl pyrrolidone (8 g PVP in 10 ml heparin solution, 100 units/ml). This solution was removed prior to sampling and the cannula washed with bacteriostatic sodium chloride (Ambot solution; Cutter Lab., Berkeley, Calif.). The day before the experiment, the rats were anesthetized with diethyl ether and the common bile duct was exposed by a 3-cm midline incision, extending from the xiphi-sternum. The bile duct was cannulated 0·5 to 1·0 cm from the liver hilum with PE-50 tubing (Intramedic polyethylene tubing, Clay Adams, Parsippany, N.J.) and securely anchored. The catheter was passed under the skin and brought out through an incision over the scapula.

Chemicals. Pregnanolone was purchased from Schwarz-Mann (New York, N.Y.); 1,2-^{3}H-pregnanolone from New England Nuclear Corp. (Boston, Mass.); β-glucuronidase Type 1, crude bacterial powder, from Sigma Chemical Corp. (St. Louis, Mo.); 2,5-diphenyloxazole (PPO), dimethyl-POPOP and Triton-X-100 from Packard Instrument Co. (Downers Grove, Ill.).

Experimental procedure. The drug administered was a mixture of pregnanolone, 20 mg, and ^{3}H-pregnanolone, 50 μCi, dissolved in 0·5 ml ethanol, to which distilled water (0·5 ml) was added dropwise to form a crystalline suspension (final sp. act. = 2·5 μCi/mg). Control blood and bile samples were collected and the bile flow rate was determined. After administration of a non-hypnotic dose of pregnanolone (40 mg/kg, i.p.), blood samples, 0·3 ml, and bile samples were collected as follows: (1) blood samples at 15-min intervals and (2) bile samples for four 5-min intervals, one 10-min, and at 15-min intervals thereafter. Depending upon the experiment, this sampling procedure was followed for a period of 1·5–6 hr. The animals were loosely immobilized in a towel while blood samples were being withdrawn or while bile flow rates were being determined; during bile collections the animals were unrestrained. In addition, bile samples were collected at random times as late as 122 hr. Duplicate 10-μl samples of bile or 100 μl of serum were added to 10 ml of a scintillation mixture consisting of 5·5 g PPO, 0·3 g dimethyl-POPOP, 667 ml toluene and 333 ml Triton X-100. Radioactivity was measured with a Packard liquid scintillation counter model 3380 equipped with a model 544 absolute activity analyzer which automatically determined and corrected for the samples' counting efficiencies which varied from 25 to 40 per cent.

Enterohepatic circulation. To determine whether or not enterohepatic circulation plays a significant role

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† Other trivial names used: 6-hydroxypregnanolone, 5β-pregnan-3α,6α-diol-20-one; 17-hydroxypregnanolone, 5β-pregnan-3α,17α-diol-20-one; pregnanediol, 5β-pregnan-3α,20α-diol; 20β-pregnanediol, 5β-pregnan-3α,20β-diol; progesterone, pregn-4-ene-3,20-dione; pregnanetriol, 5β-pregnan-3α,17α,20α-triol; 20β-pregnanetriol, 5β-pregnan-3α,17α,20β-triol; pregnanedione, 5β-pregnan-3,20-dione.

in the excretion of pregnanolone, the following procedures were used. First, bile was collected continuously over a 3-hr period from cannulated "donor" rats which had been injected intraperitoneally with the standard dose of tritiated pregnanolone. Secondly, an aliquot, 1·5 ml, of the pooled "donor" bile was administered intragastrically to cannulated "recipient" rats. Finally, bile samples were collected and bile flow rates determined under the conditions previously stated (see experimental procedure section) for 6 hr.

Extraction procedures. The cecum with its contents from rats sacrificed 6 hr after pregnanolone administration was minced into small sections and homogenized in 0·9% saline (1:2). The homogenate was centrifuged, the residue discarded and the aqueous phase extracted with 2 vol. ethyl acetate and 2 vol. diethyl ether. The extracted solutions were reduced to a small volume and partitioned between petroleum ether and 90% methanol, which removed most of the extracted unlabeled lipids. Fecal pellets collected during the experiment were similarly homogenized and extracted. Efficiency of extraction was at least 85 per cent.

After the addition of an equal volume of distilled water, serum and urine samples were acidified with 0·1 N HCl and extracted with 3 vol. diethyl ether followed by 2 vol. ethyl acetate. Each solvent extract was chromatographed and aliquots were hydrolyzed. In the case of the urine, a non-extracted sample was also chromatographed.

Thin-layer chromatography (TLC). An aliquot of bile or extract (usually 40 μl) was placed on a Silica gel thin-layer plate (5 × 20 cm, Silplate-F-52, Brinkmann Instruments, Des Plaines, Ill.) and developed to achieve maximum separation of the standards, usually 3–4 hr. The solvent system consisted of chloroform–methanol (95:5). Standards run on the same plates were detected with a vanillin reagent spray (3 g vanillin in 100 ml ethanol plus 0·25 ml concentrated sulfuric acid). The area near the origin containing polar metabolites was scraped from the plates, placed in a Pasteur micropipette, and eluted with ethanol. Aliquots of the eluent were taken for solvolysis and for treatment with β-glucuronidase, as previously described [7]. In some cases, ether extracts containing the hydrolyzed conjugates were rechromatographed in the same system. The developed plates were divided into 0·5-cm areas which were scraped and counted. Total radioactivity accounted for 80–90 per cent of the total radioactivity spotted.

Tissue. Radioactivity in tissues was measured by placing 75- to 100-mg portions into a counting vial containing Soluene-100 (Packard Instrument Co., Downers Grove, Ill.), 1 ml/100 mg, and incubated at 65° until completely digested. Before addition of the scintillation mixture, the solubilized tissue was neutralized with 1 N HCl to inhibit chemiluminescence.

RESULTS

Pregnanolone excretion and distribution. After intraperitoneal injection of the pregnanolone suspension, peak levels were found at about 30 min in both serum and bile (Fig. 1), though it could be detected in the bile within 3 min. These data refer to the apparent

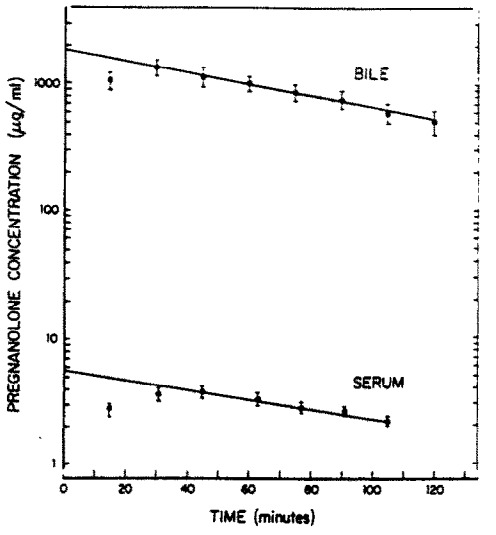

Fig. 1. Pregnanolone concentration in bile and serum after the administration of (^{3}H)-pregnanolone (40 mg/kg, i.p.). For the biliary data, each point represents the mean ± S.E.M. of four animals, while the serum data is the mean ± S.E.M. of three animals. The slopes of the two least squares regression lines for the declining phase were not statistically different. Correlation coefficients (r) = 0·99.

mean which includes pregnanolone, its metabolites and conjugates. The decline in the apparent pregnanolone concentration in the serum parallels that in the bile, indicating a constant bile to serum ratio. Data from other studies confirmed that this ratio was maintained for at least 6 hr. From the serum data for this 2-hr period, the apparent volume of distribution, *Vd*, and the apparent volume of distribution ratio, *VdR*, can be calculated from the equations: (1) Vd = dose/S_o where dose is the mean dose/animal and S_o is the extrapolated mean serum pregnanolone concentration; and (2) $VdR = Vd/w$ where w is the mean weight of the animals.

From these equations *Vd* was 1·22 liters and *VdR* was 3·58 liters/kg. These figures indicate the extensive tissue distribution of pregnanolone.

From the concentration of pregnanolone in serum, liver and bile found at 60 and 105 min after administration (Table 1), the concentration gradient established from serum to bile was calculated to be approximately 400. In a single study, the urinary concentration of pregnanolone at 90 min was about 70 times greater than that of the serum, a level intermediate between the liver and biliary concentrations relative to serum.

The mean bile flow rates, pregnanolone concentration and biliary excretion of pregnanolone (concentration × bile flow rate), during a 6-hr collection

Table 1. Relationship between apparent pregnanolone concentrations in serum, liver and bile

Time of sacrifice	60 min	105 min
Serum concn (μg/ml)	3·08 ± 0·09*	1·97 ± 0·07*
Liver concn (μg/g)	40·2 ± 11·8	71·8 ± 21·2
Bile concn (μg/ml)	1240 ± 16	798 ± 25
Liver/serum ratio	13·1 ± 4·2	36·4 ± 11·0
Bile/serum ratio	403 ± 17	405 ± 27

* Mean ± S.D. of two animals.

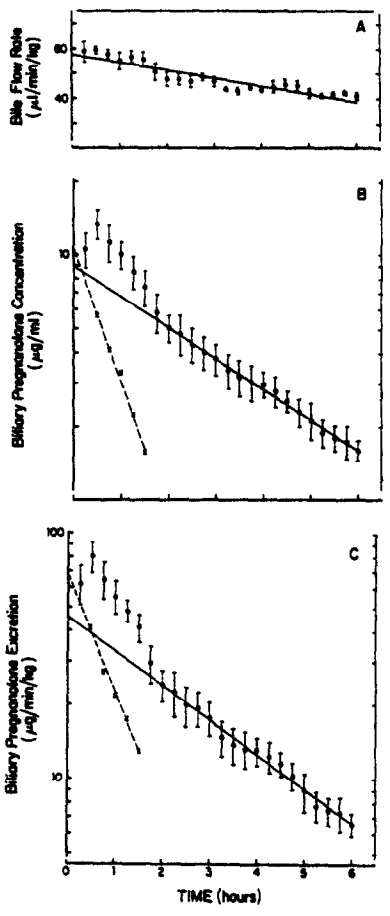

Fig. 2. Bile flow rate, biliary pregnanolone concentration, and biliary pregnanolone excretion for 6 hr after the administration of (^{3}H)-pregnanolone (40 mg/kg, i.p.). Each point represents the mean ± S.E.M. of four animals. (A) Regression line for the decline in the mean biliary flow rate (μl/min/kg) (r = 0·92). (B) Regression analysis for the decline in the mean biliary pregnanolone concentration (μg/ml). The solid line (●——●) is the regression line for the period of decline from 120 to 360 min (r = 0·99). The dashed line (× --- ×) is the regression line for the period 30–90 min for the differences between the experimental and extrapolated values contributed by the later period (120–360 min) (r = 0·99). (C) Regression analysis of the decline in the mean biliary pregnanolone excretion (μg/min/kg). The solid line is the regression line for the period 120–360 min (r = 0·99), while the dashed line is the regression line for the period 30–90 min for the difference between the experimental and extrapolated values contributed by the later period (120–360 min) (r = 0·99).

period, are presented in Fig. 2. There was a gradual decline in the biliary flow rate during this collection period despite periodic replacement with isotonic saline to compensate for blood and bile losses. Based on results from a linear regression analysis, the decrease in the mean biliary flow was 0·054 μl/min/kg so that the cumulative decrease during the 6-hr period was 33 per cent (57·8 to 38·7 μl/min/kg). The biliary concentration of pregnanolone reached a maximum of 1340 μg/ml about 30 min after adminstration. Semi-logarithmic plots of either mean biliary concentration or mean biliary excretion revealed that the decline was the sum of two first-order processes. The period of decline can, therefore, be fitted to a differential equation of the form

$$C = Ae^{-\alpha t} - Be^{-\beta t}, \quad (1)$$

where the first term describes the rapidly declining first phase (dashed lines in the figures) and the second term describes the slowly declining second phase. The equation for the decline in the mean biliary pregnanolone concentration, c, was:

$$c(\mu g/ml) = 1081e^{-0.0210t} - 893e^{-0.0048t} \quad (2)$$

while the equation for the decline in biliary excretion of pregnanolone, e, was:

$$e(\mu g/min/g) = 68{\cdot}7e^{-0.0186t} - 45{\cdot}9e^{-0.0054t}. \quad (3)$$

The biliary elimination half-life (the time required for the pregnanolone concentration in the bile to decrease by one-half) is associated with the rate constant β according to the equation $T_{1/2} = 0{\cdot}693/\beta$. Since β is a first order rate constant, the $T_{1/2}$ is a constant value independent of the initial concentration. However, this $T_{1/2}$ only accurately describes the biliary elimination half-life at times when $Be^{-\beta t} \gg Ae^{-\alpha t}$. This will approximately be the case at 180 min after administration when $Be^{-\beta t} = 10\ Ae^{-\alpha t}$. At this and later times, the biliary elimination half-life was 129 min. Over the 6-hr period, about 20 per cent of the administered dose was excreted in the bile. Despite this early rapid excretion, radioactivity was still detectable 5 days post-injection in rats with continuous biliary diversion to prevent enterohepatic cycling.

Metabolites in the bile. Analyses by thin-layer chromatography of a bile sample are shown in Fig. 3.

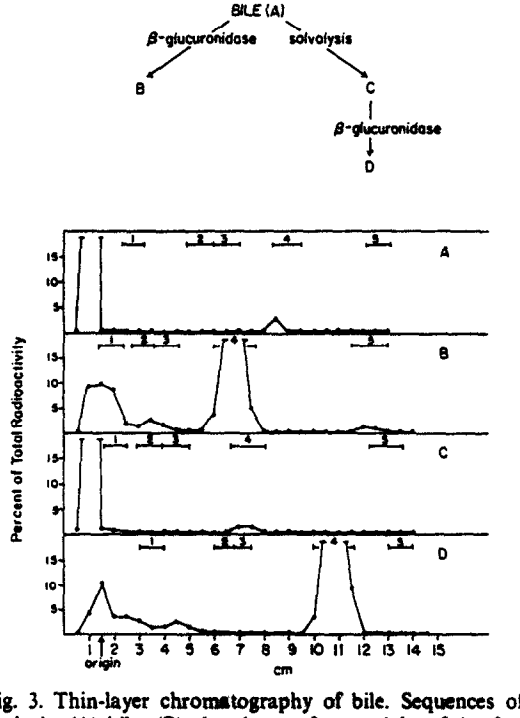

Fig. 3. Thin-layer chromatography of bile. Sequences of analysis: (A) bile; (B) eluted area from origin of A after β-glucuronidase treatment; (C) eluted area from origin of A after solvolysis; and (D) eluted area from origin of C after β-glucuronidase treatment. Standards run on each plate were: (1) 6β-hydroxypregnanolone; (2) 17α-hydroxypregnanolone; (3) 20β-pregnanediol; (4) pregnanolone; and (5) pregnanedione.

Ninety per cent of the radioactivity remained at the origin and about 4 per cent was in the region of free pregnanolone (Fig. 3A). The non-migrating area at the origin was eluted and aliquots were treated with β-glucuronidase or treated by solvolysis to hydrolyze sulfate esters. After chromatography of the glucuronidase-treated sample, 18 per cent of the total radioactivity on the plate remained at the origin, while 68 per cent was recovered as free pregnanolone (Fig. 3B). In contrast, repeat chromatography of the solvolyzed aliquot revealed that 96 per cent of total radioactivity remained at the origin (Fig. 3C), indicating that sulfate esters were minimal or absent. After glucuronidase treatment of the non-migrating area from the solvolyzed sample, a prominent free pregnanolone peak was found (Fig. 3D), confirming that this represented the glucuronide ester.

Bile samples were treated with β-glucuronidase, subjected to thin-layer chromatography, and the percentage of radioactivity in various areas of the chromatogram corresponding to compounds which (a) remained at origin, (b) migrated but had an R_f less than that of pregnanolone, (c) migrated as pregnanolone, or (d) had an R_f greater than that of pregnanolone, was determined. Radioactivity at the origin accounted for approximately 13 per cent of the total, in the region between the origin and pregnanolone, 24 per cent, pregnanolone, 60 per cent, and in the area of R_f greater than that of pregnanolone, 3 per cent (Fig. 4). Differences within each of the four areas at the various collection times appeared to be minor, indicating lack of a stepwise biotransformation resulting in accumulation of a particular metabolite in the bile.

In addition to migration, identification of pregnanolone and pregnanediol was verified by carrier recrystallization to constant specific activity [8]. Negative results were obtained with 17-hydroxypregnanolone, 20β-pregnanediol, pregnanetriol and 20β-pregnanetriol (data not shown).

Metabolites in the serum. Chromatography of both ether and ethyl acetate extracts of serum obtained at 90 min revealed that most of the radioactivity migrated as free pregnanolone with a small peak remaining at the origin. This pattern of migration was the reverse of that seen in the bile. Treatment of the material at the origin with β-glucuronidase

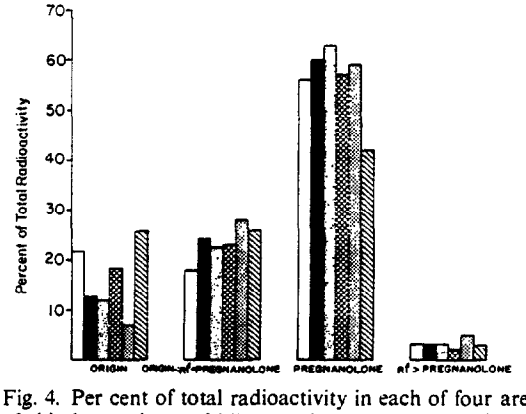

Fig. 4. Per cent of total radioactivity in each of four areas of thin-layer plates of bile samples taken at 0·25 (□), 1·5 (■), 3 (□), 6 (▩), 51 (▨) and 122 (▧) hr after i.p. administration of pregnanolone. The bile samples were subjected to β-glucuronidase before chromatography.

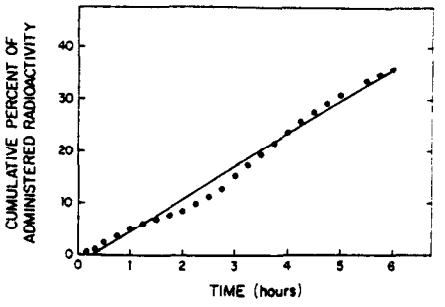

Fig. 5. Cumulative per cent of the biliary excreted pregnanolone appearing in the bile for 6 hr after intragastric administration of 1·5 ml bile donated by cannulated rats injected with (^{3}H)-pregnanolone (40 mg/kg. i.p.) (r = 0·99).

and rechromatography revealed a peak consistent with pregnanolone.

Metabolites in the urine. Urine obtained at 90 min was similarly extracted and the sample and the extracts were treated to hydrolyze both glucuronide and sulfate conjugates. No free pregnanolone was found in the urine, nor were any glucuronide conjugates present, in sharp contrast to the bile where pregnanolone glucuronide was always present in large amounts. About 30 per cent of the radioactivity migrated from the origin after solvolysis; no single metabolite predominated.

Intragastric administration of bile—the enterohepatic cycle. To determine whether pregnanolone entered into an enterohepatic cycle, bile collected from donor rats was administered into the stomach of cannulated recipient rats. The cumulative per cent of pregnanolone excreted in the bile increased during a 6-hr collection period (Fig. 5). As was the case for intraperitoneally administered pregnanolone, there was almost immediate appearance of radioactivity after intragastric administration of donor bile.

The concentration of radioactivity in the bile followed a biphasic pattern with an initial maximum at 30 min, followed by a decline, with a second higher peak at 210 min (Fig. 6). This appears to explain the sigmoidal cumulative excretion curve (Fig. 5); therefore, no reliable rate constants for biliary elimination could be determined. The initial excretory peak was probably due to rapid absorption of unconjugated pregnanolone present in the administered bile. This proposal was strengthened by the finding that the total amount of pregnanolone excreted in this initial

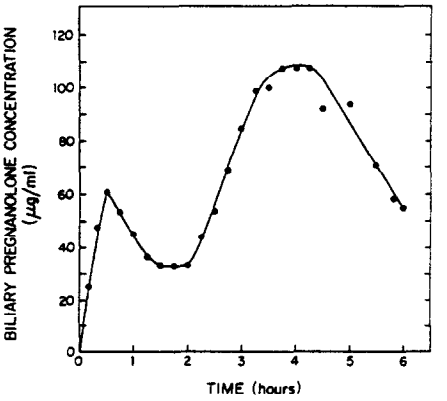

Fig. 6. Radioactivity in bile of rat after intragastric administration of donor bile at time zero.

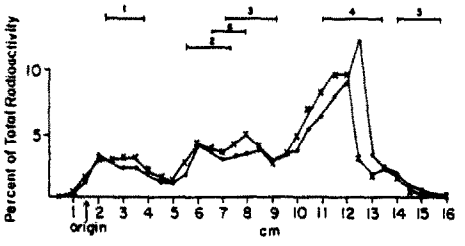

Fig. 7. Chromatographic profile of extract feces (●——●) and cecum (×——×). Standards as in Fig. 3 plus (6) pregnanediol.

peak (51 μg) corresponded closely to the amount of free pregnanolone present in the administered bile (69 μg). The appearance of the second peak was consistent with an enterohepatic cycle, i.e. hydrolysis of the glucuronide ester, absorption, uptake by the liver and reconjugation. At the dose given, about one-third of the material administered in the donor bile was excreted in the bile of the recipient in a 6-hr period. The pattern of metabolites was similar to that found after i.p. administration of pregnanolone, i.e. primarily pregnanolone glucuronide.

At sacrifice, 6 hr after intragastric administration, no bile remained in the stomach and almost none was seen in the small intestine. Total radioactivity in the cecum and feces was about 25 per cent of the dose. Total radioactivity determined from solubilized intestinal segments decreased along the length of the small intestine. Four equal-length sections from duodenum to cecum contained 50, 37, 7 and 6 per cent of the radioactivity found localized in the small intestine respectively. These data, plus the observation that radioactivity rapidly appeared in the bile after intragastric administration, indicate that pregnanolone was absorbed in the small intestine.

The feces and homogenized cecum were extracted to determine if hydrolysis of the conjugated steriods occurred within the gastrointestinal tract. In contrast to the donor bile (Fig. 3), TLC of the cecal and fecal extracts revealed that less than 5 per cent of the total radioactivity remained at the origin. The fecal and cecal extracts displayed similar chromatographic profiles (Fig. 7). Other differences between these extracts and the donor bile were that the extracts contained less than 35 per cent of total radioactivity as free pregnanolone, 28 per cent as the 20β-diol and the remaining 37 per cent as more polar, nonconjugated metabolites. The presence of 17α-hydroxypregnanolone, both 20α- and 20β-diols, and 6β-hydroxypregnanolone in the feces was confirmed by carrier recrystallization. Since the latter compounds were present either in extremely low concentrations, or more probably were absent, in the bile of the donor, it would appear that they had been formed in the intestine.

DISCUSSION

The present data indicate that pregnanolone appeared in the serum within minutes after intraperitoneal administration of a crystalline suspension of pregnanolone or after intragastric administration of bile derived from a pregnanolone-pretreated animal. The liver avidly concentrated the steroid (10- to 36-fold) and rapidly excreted it, mainly as the glucuronide conjugate of non-metabolized pregnanolone. Although the $T_{1/2}$ for the decline in the pregnanolone excretion in the bile was of the order of 2 hr, detectable radioactivity was found in the bile for many days post-injection, despite continuous biliary diversion. Excretion would be expected to be even more prolonged in intact animals because of enterohepatic recycling.

Hepatic microsomes supplied with an NADPH-generating system form a number of pregnanolone C6, 17 and 20 hydroxy metabolites [8], but these were found only in minor amounts in the bile. Surprisingly, little pregnanediol was found in the bile or urine, whereas this compound is commonly considered to be the major metabolite of progesterone, and is found in high concentrations in the urine and bile of man [9, 11].

Our data suggest that pregnanolone glucuronide was hydrolyzed in the intestine, and pregnanolone absorbed as the free steroid. Although secretion into the intestine of circulating metabolites could not be ruled out, it seems probable that reduction of C20 and possibly hydroxylation at C6 and 17 took place in the intestine. In a series of papers, Gustafsson and Eriksson [12] have shown important differences in the biliary, urinary and fecal excretion of metabolites of progesterone and corticosterone in germ-free and conventional rats. Our data do not allow differentiating between biotransformations which occurred via enteric microflora vs those via intestinal mucosal enzymes.

The high bile:serum ratio for pregnanolone (about 400) found in this study far exceeds that reported after administration of phenobarbital (10), pentobarbital (22), bilirubin (30), indocyanine green (30) and chlorthiazide and BSP (80–90) [1, 2]. The isolated rat liver perfused with progesterone, testosterone and other steroids concentrated metabolites to about 1000 times over that of the parent compound in the perfusion fluid [13]. Thus, the biliary concentration gradient may be greater for steroids than for drugs, though further studies are needed to determine if this is a valid generalization.

Pregnanolone and/or its metabolites persisted in the animal for many days after a single injection, due to deposition in the liver [8] and enterohepatic cycling (present study). Since lower rates of hydroxylation [7] and of bile flow exist in the newborn, persistence of pregnanolone and/or related steroids in the liver can be expected to be even greater than that found in the adult. Since pregnanolone and structurally similar pregnanes are potent inhibitors of model hepatic drug-metabolizing reactions [14, 15], it appears possible that the persistence of pregnanolone and/or related steriods may contribute to the impairment of hepatic drug metabolism seen in the newborn of many species.

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