PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 162, 495–502 (1979)

Hepatic Metabolism of Glycodehydrocholic Acid in the Dog and Rat (40710)[1, 2]

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Dehydrocholic acid (3,7,12-triketocholanic acid, DCA, **Ia**, Fig. 1), a bile acid analog, is a potent choleretic. It has been assumed that choleresis is due to the osmotic effect of DCA transported into bile. Since DCA does not form micelles, if it or a derivative were excreted unchanged, the compound would be of great value in exploring the mechanisms by which taurocholate, a micelle-forming bile acid, enhances transport of various organic anions into bile (1–3).

Evidence for hepatic metabolism of DCA has been obtained, however, by several investigators (4–11). In the most extensive study, Soloway and associates (7) found that DCA (**Ia**) was totally metabolized in man, and bile contained reduced metabolites **II**, **III**, and **IV** conjugated with taurine (Fig. 1). The ratio of products **II**, **III**, and **IV** was 20:70:10. Cholic acid (**IVa**) readily forms micelles. Compounds **IIa** and **IIIa** are much less effective in micelle formation but they do form mixed micelles with **IVa.**

Young and Hanson (12) provided evidence that glycodehydrocholic acid (GDCA, **Ib**) was protected from metabolism. In isolated rat liver perfusion experiments, they reported that GDCA was excreted unchanged into bile. GDCA did not form micelles and did not enhance the biliary excretion of the phospholipid phosphatidylcholine. By contrast, taurocholate (**IVc**) increased phosphatidylcholine excretion by 60% in the perfused rat liver.

We sought to determine if GDCA is altered *in vivo* by injecting tritiated GDCA and examining the tritiated compounds excreted into bile of the intact dog and rat. For comparison, DCA was also studied in several dogs.

Materials and methods. Chemicals. Sodium taurocholate, grade A, sodium glycodehydrocholate, grade A, and glycocholic acid (**IVb**) were purchased from Calbiochem, La Jolla, California; cholic acid (**IVa**), 5β-cholanic acid-3α,7α-diol-12-one (**IIIa**), 5β-cholanic acid-3α,12α-diol-7-one (**IIIa**), and 5β-cholanic acid-3α-ol-7,12-dione (**IIa**) from Steraloids, Inc., Winton, New Hampshire; dehydrocholic acid (**Ia**) and glycine ethyl ester hydrochloride from Sigma Chemical Company, St. Louis, Missouri; triethylamine from Matheson, Coleman and Bell, Norwood, Ohio; and EEDQ (*N*-ethoxycarbonyl-2-dihydroquinoline) from Aldrich Chemical Company, Milwaukee, Wisconsin. All were used as supplied except dehydrocholic acid which was recrystallized once from acetone. The steroid compounds all were at least 95% pure by thin-layer chromatography (tlc). Amberlite XAD-2 (Mallinckrodt Chemical Co., St. Louis, Mo.) was washed with methanol and stored under water.

[^{3}H]Glycodehydrocholic acid was obtained from New England Nuclear Corporation as a solution in ethanol. It was purified by tlc twice in solvent system 1 (see below) to give 170 μCi of 90% radiopure material. In solvent system 2 the impurities appeared as a broad, low-intensity band extending from the origin to the main peak, and in solvent system 1 some scans showed a small shoulder on the leading edge of the single peak. Evidently there were several small impurities, but no discrete peak corresponding to any of the compounds studied. The material weighed <5 mg, and it was stored as a solution in ethanol (12 μCi/ml). It had the same R_f as authentic glycodehydrocholic acid by tlc in our 2 solvent systems.

Glyco-5β-cholanic acid-3α,7α-diol-12-one (**IIIb**) was prepared by a procedure based on that of Tserng *et al.* (13) for synthesis of

[1] These studies were supported by Grant PO1 AM19329 from the National Institutes of Health.

[2] Part of these studies were reported in abstract form in Gastroenterology **75**, 980 (1978).

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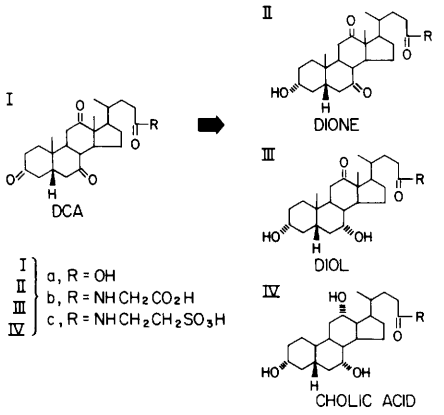

FIG. 1. Triketo bile acid metabolism.

glycine and taurine conjugates of bile acids. 5β-Cholanic acid-3α,7α-diol-12-one (**IIIa**, 80 mg, 0.197 mmole) with EEDQ (68.2 mg, 0.276 mmole) and glycine ethyl ester hydrochloride were converted into the corresponding glyco-diol-ethyl ester, 29.2 mg (61% yield). A second recrystallization gave 39 mg of crystals, pure by tlc. The ester (39 mg, 0.079 mmole) was saponified in hot ethanolic K_2CO_3 to give the glyco-diol **IIIb** as a white powder, 32 mg (89% yield). Thin-layer chromatography (system 2, see below) showed one spot (R_f 0.06).

Glyco-5β-cholanic acid-3α-ol-7,12-dione (**IIb**) was synthesized by a procedure similar to that used for preparation of glyco-diol **IIIb** above. 5β-Cholanic acid-3α-ol-7,12-dione (**IIa**, 100 mg, 0.247 mmole) was converted to the glyco-dione-ethyl ester (60 mg, 50% yield) obtained as a white solid from ethyl acetate–hexane. Thin-layer chromatography showed one spot (system 1, R_f 0.39; system 2, R_f 0.61). Saponification of this ethyl ester (60 mg, 0.122 mmole) gave glyco-dione **IIb** as a white powder (30 mg, 53% yield) pure by tlc (system 1, average R_f 0.32; system 2, average R_f 0.14).

Animal experiments. 1. Dog. Adult mongrel dogs weighing 15.5 to 18.6 kg were fasted for 12 hr prior to induction of anesthesia with intravenous pentobarbital (30 mg/kg). The cystic duct was ligated and common duct cannulated (PE-190) through a midline laparotomy incision. The renal pedicles were ligated in dogs 1 and 2. Body temperature was maintained at 37–38°C by heating pads under the dogs. Solutions were infused through PE-90 tubing in each jugular vein with a Bowman pump at rates of 1–1.5 ml/min. Infusion solutions of DCA–Na^+ were prepared by dissolving DCA and an equivalent amount of NaOH in pH 8 phosphate buffer (0.1 *M*) and diluting with normal saline; and GDCA–Na^+ solutions were prepared by dissolving the salt in normal saline.

Table I summarizes these experiments. In all dogs except dog 1, a constant taurocholate infusion was maintained through one jugular vein in order to replace bile acids lost in the interrupted enterohepatic circulation and thereby stabilize background bile acid excretion and bile flow. In each experiment after a period of stabilization of at least 30 min, a control bile sample was collected for 10 to 15 min before infusions or injections of GDCA or DCA were administered. Bile samples were collected in tared vials over 5- to 10-min periods.

2. Rat. Two adult Holtzman rats weighing 175 and 350 g were anesthetized with pentobarbital (30 mg/kg) injected intraperitoneally and the common bile duct was cannulated (PE-10). Bolus injections of GDCA–Na^+ in normal saline were made through a PE-50 cannula in the femoral vein. Injection volumes were $\leq$ 1 ml. Bile was collected for 5- to 10-min periods in tared vials before and after doses of GDCA. The experiments are summarized in Table I.

Analytical procedures. Bile volume was measured gravimetrically assuming a density of 1.0 g/ml. Bile salt concentrations in bile were determined by a 3α-hydroxysteroid dehydrogenase (Worthington Biochemical Corp.) method (14). DCA (**Ia**) and GDCA (**Ib**) did not react with the enzyme. Dione (**IIa**), glyco-dione (**IIb**), diol (**IIIa**), glyco-diol (**IIIb**), and cholic acid (**IVa**) as well as its glycine (**IVb**) and taurine (**IVc**) conjugates reacted similarly in the assay. Sodium and potassium concentrations in bile were measured by flame photometry (IL flame photometer, Model 143, Instrumentation Laboratory, Inc.), and chloride concentrations were determined by titration with a silver electrode (Buchler–Cotlove Chloridometer, Buchler Instruments, Inc.). Bicarbonate ion concentrations in bile were determined by the number of milliequivalents of HCl neutralized by an aliquot of the bile sample (15).

Thin-layer chromatography was per-

TABLE I. SUMMARY OF GLYCODEHYDROCHOLATE AND DEHYDROCHOLATE STUDIES IN THE DOG AND RAT.

Animal No.	Body wt	Liver wt (g)	Compound administered	How administered	Dose (μmole/kg)	^{3}H dose (μCi)	Background taurocholate infusion (μmole/kg/min)
1. Dog studies							
1a	15.5 kg	475	[^{3}H]GDCA-Na$^+$	Bolus	Trace	24	—
b			[^{3}H]GDCA-Na$^+$ + GDCA-Na$^+$	Bolus	13.4	25	—
2a	18.6 kg	437	GDCA-Na$^+$	Bolus	49.6		0.16
b			DCA-Na$^+$	Bolus	49.6		0.16
3a	18.2 kg	625	GDCA-Na$^+$	Constant infusion	0.42/min		0.31
b					1.70/min		0.31
c					4.26/min		0.31
4a	18.6 kg	441	DCA-Na$^+$	Constant infusion	0.37/min		0.26
b					1.46/min		0.26
c					3.66/min		0.26
2. Rat studies							
1	350 g		[^{3}H]GDCA-Na$^+$	Bolus	Trace	12.3	—
2a	175 g		[^{3}H]GDCA-Na$^+$ + GDCA-Na$^+$	Bolus	50	0.63	—
b					100	1.26	—
c					300	3.78	—

formed on 0.2-mm-thick silica gel plates without fluorescent indicator (EM reagents). Five-microliter samples of bile or solutions were applied and plates were developed with the following solvent systems: 1, isoamylacetate–propionic acid–*n*-propanol–H_2O, 40:30:20:10 (16); or 2, chloroform–methanol–acetic acid, 40:4:2 (17). Spots were visualized by spraying the plates with anisaldehyde reagent (18) and heating at 100°C for 10 min. Samples were dissolved in 10 ml of Biofluor scintillation cocktail (New England Nuclear) in 20-ml screw-cap glass vials and counted in a Packard liquid scintillation spectrometer, Model 3255.

Method A was used to identify tritiated metabolites in bile from animals injected with trace weights of [^{3}H]GDCA with high specific activity (dog 1a and rat 1). The bile sample containing the highest radioactivity (dog 1a, 3.1 ml, 10.9 μCi; rat 1, 0.7 ml, 8.4 μCi) was diluted to five times its original volume with water and acidified to pH 3.5 with 0.5 *N* HCl. This solution was slowly passed through a 5-g Amberlite XAD-2 column to absorb the bile salts. The column was washed with water until the eluant was neutral and then bile salts were eluted with absolute methanol. In separate studies [^{3}H]GDCA alone, or after addition to bile, was removed by the Amberlite column and subsequently was completely eluted with absolute alcohol. Evaporation of methanol under aspiration left a trace of residue which contained 87–100% of the starting radioactivity. Aliquots of the Amberlite-purified residue containing 0.5 to 2 μCi of tritium were streaked over 2 cm at the origin of tlc plates which were then developed in solvent systems 1 and 2. Standard compounds **Ib** to **IVb** were spotted on the plates for comparison. Spots were visualized with anisaldehyde reagent and plates were scanned with a Packard radiochromatogram scanner, Model 7201, to locate tritium activity. A second set of tlc's were run on samples to which authentic [^{3}H]GDCA was added (peak enhancement experiments). Peak ratios were measured from scans of plates run in solvent system 2. Attempts to apply enough unprocessed bile to the tlc plates to obtain an adequate scan were unsuccessful since severe tailing of bands resulted.

Method B was used to measure ratios of tritiated metabolites in bile from animals injected with a mixture of tritiated and cold GDCA (dog 1b and rat 2). Bile containing the highest tritium activity was applied at the origin of the tlc plate in four spots (5 μl each) and compounds **Ib** to **IVb** were spotted for comparison. Plates were developed in system 2 and spots visualized with the anisaldehyde reagent. Bands corresponding to the reference compounds were cut and leached with 1 ml methanol in 20-ml vials for 24 hr. Then 10 ml of scintillation cocktail was added and vials were counted.

GDCA metabolites in bile in dog 3 were estimated semiquantitatively as follows: A bile sample near the end of each of the three infusion periods was analyzed. A set of ten 1: 1 serial dilutions was prepared for each bile sample and for standard solutions of GDCA-Na^+ and glycocholic acid. Then 5 μl of each dilution was spotted on a tlc plate. Reference compounds **Ia** to **IVa** were also spotted for comparison. Plates were developed in system 2 and spots visualized with anisaldehyde reagent. The size and the intensity of the color of the spots of compounds excreted into bile were related visually to those of known concentrations of the standards. The limit of detection for GDCA and glycocholic acid was similar (0.2 nmole). Bile from each infusion 3a to 3c contained approximately equal amounts of glycocholic acid and GDCA.

Results. Injection of trace molar amounts of [^{3}H]GDCA into rat 1 resulted in excretion of 81% of the label in bile in 25 min. Similar injection of [^{3}H]GDCA into dog 1 (1a) led to recovery of 56% of the label in bile in 60 min. These bile samples were analyzed by tlc after isolation of bile salts by Amberlite XAD-2 column purification (see method A above). Thin-layer chromatography (Fig. 2) showed two peaks in system 1 and three peaks in system 2 but no unchanged GDCA was present in bile. Peak enhancement with authentic [^{3}H]GDCA in system 1 confirmed the absence of GDCA. Since the most likely metabolites were the reduced compounds **IIb**, **IIIb**, and **IVb** which are glycine conjugates of the compounds known to be formed from DCA (7), we synthesized glycine conjugates **IIb** and

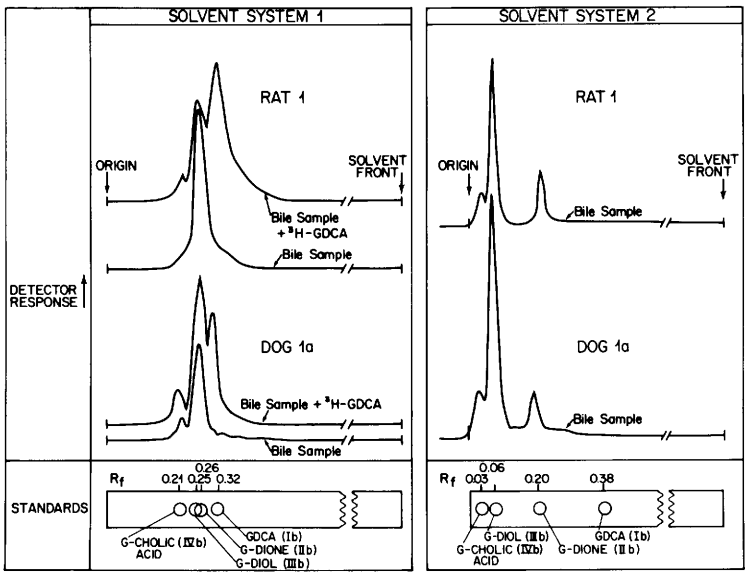

FIG. 2. Chromatograms of GDCA metabolites in bile. Radio-tlc of bile from rat 1 and dog 1a given [^{3}H]GDCA by vein in trace molar doses. Bile samples processed by Method A were run in tlc systems 1 (left panel) and 2 (right panel), respectively. Two radioactive peaks in bile were identified in system 1 and three peaks in system 2. Addition of [^{3}H]GDCA to bile resulted in the appearance of a new radioactive peak in system 1. The original two peaks in bile were not enhanced by [^{3}H]GDCA. System 2 provides better separation of the radioactive compounds excreted in bile. The three peaks in system 2 have mobilities identical with those of standard compounds **IIb** (G-dione), **IIIb** (G-diol), and **IVb** (G-cholic), respectively.

IIIb from the commercially available bile acids **IIa** and **IIIa** by the coupling procedure of Tserng *et al.* ((13); see Materials and Methods). The synthetic glyco-dione **IIb** and glycodiol **IIIb** exhibited identical tlc mobilities to those of the two major metabolites in bile, and the third metabolite corresponded to glycocholic acid (**IVb**) by tlc R_f value. System 2 provided much better separation of **IIb** and **IIIb** than system 1, explaining why only two metabolite peaks were found in system 1, but three peaks in system 2. Ratios of peak areas from scans of plates developed in solvent system 2 gave the relative amounts of metabolites (Table II). The ratios of reduced metabolites **IIb–IVb** in bile were similar in dog 1a and rat 1.

Metabolites were not isolated and characterized. Structure assignments were made by analogy to the work of Soloway *et al.* (7) and similarity of tlc mobilities to those of authentic compounds. Since glyco-diol **IIIa** and the isomeric 3,12-diol are not separated by our solvent systems and the corresponding glycine conjugates are probably not separated, we were unable to distinguish these isomeric compounds as metabolites. Ostrow and associates (19) recently reported excretion of the 3,12-diol instead of the 3,7-diol in the bile of a rat infused with taurodehydrocholate.

Intravenous boluses of mixtures of tritiated and cold GDCA were also injected into dog 1 (1b) and rat 2. Bile from these animals was analyzed for metabolites by method B (see above). These experiments showed the appearance of small amounts of unchanged GDCA (**Ib**) in bile when the injected dose was 13 μmole/kg or greater (Table II). The relative proportion of glycocholic acid (**IVb**) in bile remained fairly constant regardless of dose, but that of glycodiol **IIIb** (or its 3,12 isomer) decreased while that of glyco-dione **IIb** increased with increasing administered doses of GDCA.

TABLE II. RELATIVE PERCENTAGE OF GDCA METABOLITES IN BILE MEASURED BY tlc AFTER INTRAVENOUS ADMINISTRATION OF [^{3}H]GDCA IN THE RAT AND DOG.

	Rat 1	Rat 2			Dog 1	
Experiment		a	b	c	a	b
Dose of GDCA (μmol/kg)	Trace	50	100	300	Trace	13.4
Method[a]	A	B	B	B	A	B
Metabolite						
Ib	0	3	3.3	4.2	0	3
IIb	15	59	47.7	62.1	9	48
IIIb	81	35	42.6	29.1	86	38
IVb	4	3	6.4	4.6	5	11

[a] A, Relative percentage of metabolites determined by radio-tlc scan of Amberlite XAD-2 isolated compounds. B, Relative percentage determined by elution and scintillation counting of spots from direct tlc of bile.

In dog 2 equal molar boluses of GDCA and DCA produced very similar choleretic effects (Fig. 3). The 3α-hydroxysteroid assay also showed similar excretion patterns. Thin-layer chromatography (solvent system 2) showed only small amounts of GDCA in bile from experiment 2a and a trace of DCA in bile from experiment 2b. Thus the excretion of 3α-hydroxymetabolites after injection of GDCA and DCA appeared similar. This is support for extensive metabolism of GDCA as has been reported for DCA (7) with the formation of 3α-hydroxymetabolites.

Constant infusion of GDCA (dog 3) and DCA (dog 4) into dogs also gave similar choleretic responses (Figs. 4 and 5). Bile samples were analyzed for 3α-hydroxysteroid compounds and for electrolytes for calculation of anion gap ($Na + K - Cl - HCO_3$). If appreciable unchanged GDCA or DCA, which are anions not measured by 3α-OH steroid assay, appeared in bile, there would be a discrepancy between 3α-hydroxysteroid excretion and anion gap excretion. Figures 4 and 5 show that the 3α-OH bile salt excretion approximates the organic anion excretion reflected by anion gap. This technique is consistent with extensive reductive metabolism of both GDCA and DCA to 3α-hydroxymetabolites. Thin-layer chromatography of bile samples from dog 4 showed only traces of DCA. The taurine-conjugated DCA metabolites in bile from dog 4 were not separated from intrinsic taurine-conjugated bile salts by our tlc systems. Bile from dog 3 showed spots corresponding to authentic **IIb**, **IIIb**, and **IVb** and a small definite spot for unchanged GDCA. The amount of GDCA in bile of dog 3 was estimated by a semiquantative dilution technique using tlc. This showed the amount of GDCA was small relative to glyco-diol **IIIb** and was approximately equal to the glycocholic acid (**IVb**) present in bile. Thus DCA was essentially completely reduced and con-

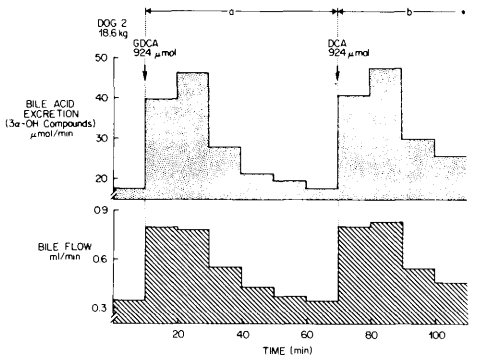

FIG. 3. Bile acid excretion and bile flow after administration of GDCA and DCA. Bolus injections of the respective compounds were administered in a dose of 924 μmole as indicated. Comparable amounts were excreted in bile over the ensuing periods (668 μmole, 72% of injected in 60 min for GDCA; 725 μmol, 79% of injected in 40 min for DCA).

jugated with taurine, whereas when GDCA was administered a small but definite amount of GDCA was excreted unchanged.

Discussion. Metabolism of GDCA was studied in dogs and rats given [^{3}H]GDCA and cold GDCA in various doses. Bile was examined by tlc for metabolites since GDCA- and glycine-conjugated metabolites are well separated by tlc from intrinsic taurine-conjugated bile salts in the dog and rat. The efficient separation of glycine conjugates in solvent system 2 allowed measurement of compound ratios directly by radio-tlc without tedious chemical conversions and possible alteration of compound ratios.

We found complete metabolism of GDCA at low doses and found 3 compounds which correspond to 3α-hydroxymetabolites **IIb**-**IVb** by tlc. We prepared glyco-dione **IIb** and

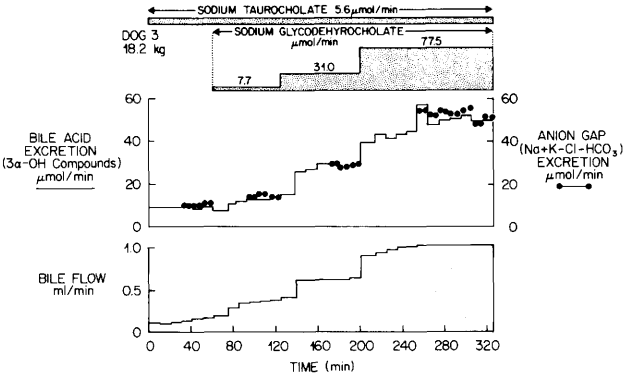

FIG. 4. Bile acid excretion and bile flow during infusion of glycodehydrocholate. Sodium glycodehydrocholate was infused at three separate constant infusion rates as indicated. Bile acid excretion based on measurements of 3α-OH compound excretion in bile and that based on measurements of anion gap excretion are virtually identical.

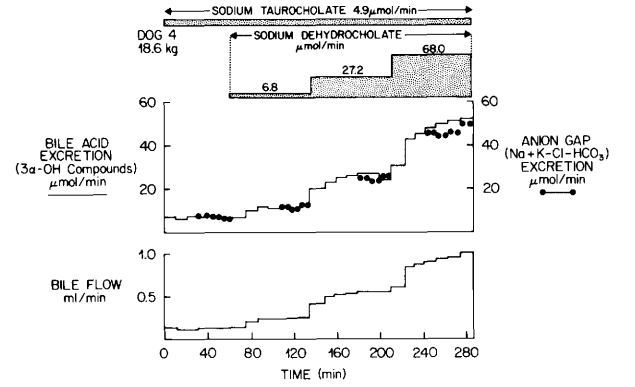

FIG. 5. Bile acid excretion and bile flow during infusion of dehydrocholate. The design of the experiment and the presentation of the data are similar to those of the experiment shown in Fig. 4.

glyco-diol **IIIb** by the coupling procedure of Tserng *et al.* (13) for tlc comparison. Our results are analogous to those of Soloway *et al.* (7) for metabolism of DCA. These authors intravenously infused ^{14}C-labeled DCA at low doses (~10 μmole/kg) and metabolites excreted in bile as taurine conjugates were deconjugated, esterified, and identified by tlc comparison with authentic methyl esters of **IIa-IVa.** Reduction of keto groups in DCA appeared to occur sequentially at C3, then C7, and finally C12 positions. Random reduction of DCA or GDCA could produce 26 possible hydroxymetabolites, and other hydroxylation products are also possible. However, the unavailability of all these compounds precluded our rigorously excluding them as possible metabolites. Metabolites **IIb** and **IIIb** were not isolated from bile but there was identical tlc mobility of the three metabolites of GDCA and compounds **IIb-IVb** in two tlc solvent systems. We could not distinguish diol **IIIb** from the isomeric 3,12-diol by our tlc systems. In a recent preliminary report, Ostrow and associates demonstrated that the 3,12 diol appears in bile of the rat following administration of taurodehydrocholate (19). This further supports our contention that **IIIb** may be the 3,7 or 3,12 isomer. However, the similarity of our product ratios of GDCA metabolites with those of Soloway *et al.* for DCA metabolism (20:70:10, **IIa:IIIa:IVa**) indicates the two compounds are handled similarly on passage through the liver.

Doses of GDCA above 13 μmole/kg in the dog and rat resulted in biliary excretion of small amounts of unchanged GDCA (Table II). Increasing doses of GDCA resulted in decrease in the relative amount of metabolite diol **IIIb** (or its 3,12 isomer) and proportionate increase in metabolite dione **IIb.** This may reflect relatively less efficient dihydroxy formation compared to monohydroxy formation as the load of GDCA is increased. The proportion of fully reduced metabolite **IVb** was relatively constant at all infusion rates.

Intravenous GDCA and DCA produced similar choleretic effects whether administered by bolus or constant infusion (Figs. 3–5). Thus conjugation of DCA with glycine prior to parenteral administration does not enhance the choleretic effect. The excretion of 3α-hydroxy bile salts as measured by the 3α-hydroxysteroid assay increased in a similar manner after both DCA and GDCA administration. We found DCA to be essentially completely metabolized in these experiments as previously reported by Soloway *et al.* (7). Thus the 3α-hydroxysteroid excretion reflects reductive metabolism of DCA and GDCA to a similar extent.

Bile from dogs 3 and 4 was analyzed for 3α-hydroxy bile salts and for electrolytes for calculation of anion gaps. Any appreciable excretion of unchanged GDCA or DCA should be reflected in a difference between the 3α-hydroxy assay and anion gap. However, no significant discrepancy was found in these dogs (Figs. 4 and 5). Thus in these experiments the 3α-hydroxy assay reflects excretion of similar amounts of 3α-hydroxy-metabolites after GDCA and DCA administration. Thin-layer chromatography of bile from these two experiments showed only trace amounts of unchanged DCA and small amounts of GDCA which approximated the amount of glycocholic acid (**IVb**) in the bile.

Summary. Our results demonstrate extensive metabolism of GDCA in the dog and rat by a pathway similar to that for DCA in man (7). Assignment of reduced metabolites and relative ratios was made by tlc comparison of tritiated metabolites with known compounds. Trace bolus doses of GDCA result in complete reductive metabolism, and increasing molar doses result in the excretion of only small amounts (<5%) of unchanged GDCA in bile. Thus our *in vivo* experiments are in contrast to the absence of GDCA metabolism reported in the isolated, perfused rat liver (12).

The authors are indebted to Ruthie Pettigrew and Becky Backof for invaluable technical assistance and to Jo Ellen Shockley for preparation of the manuscript.

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Received June 7, 1979. P.S.E.B.M. 1979, Vol. 162.