J. steroid Biochem. Vol. 22, No. 4, pp. 547–554, 1985
Printed in Great Britain.

0022-4731/85 $3.00 + 0.00

INFLUENCE OF MICROBIAL BILE SALT DESULFATION UPON THE FECAL EXCRETION OF BILE SALTS IN GNOTOBIOTIC RATS

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(*Received* 31 *August* 1984)

Summary—The fecal excretion of intraperitoneally injected 24-^{14}C-labeled taurocholate (TCA), taurolithocholate (TLCA) and the respective 3-sulfate esters (TCA-3-S; TLCA-3-S), were compared in germfree (GF) rats, conventional (CV) rats, and in gnotobiotic rats associated with *Clostridium* Cl-8 or this same strain Cl-8 plus the bile salt desulfating *Clostridium* S_1, respectively. TCA and TLCA were about two times more rapidly excreted by CV animals than by GF animals; the time required for 50% excretion of total label injected ($t_{1/2}$) was 6.6 days vs 14.9 for TCA, and 4.4 vs 8.9 for TLCA. In GF and in CV animals, TCA-3-S and TLCA-3-S were excreted more rapidly than their nonsulfated analogues; the $t_{1/2}$ values of TCA-3-S and TCA were 2.7 days vs 14.9 in GF rats, and 3.1 vs 6.6 days in CV animals. The $t_{1/2}$ values of TLCA-3-S and TLCA were 2.7 days vs 8.9 in GF rats, and 1.5 vs 4.4 days in CV rats. In gnotobiotic rats associated with *Clostridium* strains S_1 + Cl-8, fecal bile salts were nearly 100% deconjugated and desulfated and the 50% excretion times of TCA-3-S and TLCA-3-S approximated to those of TCA and TLCA in GF animals. $T_{1/2}$ of TCA-3-S in gnotobiotic S_1 + Cl-8 animals was 12.2 days vs 14.9 for TCA in GF animals. In gnotobiotic S_1 + Cl-8 animals the $t_{1/2}$ of TLCA and TLCA-3-S was 12.5 and 11.0 days, respectively. These results illustrate clearly the important effect the intestinal microflora has upon the metabolic half-life of bile salts. Moreover, they demonstrate that desulfation of bile salts by the intestinal microflora takes place in intestinal segments from where a certain degree of reabsorption is still possible, and thus point to the fact that microbial desulfation is an important variable in the overall elimination of bile salts.

INTRODUCTION

In the rat, sulfation of bile salts takes place both in the liver and in the kidney [1]. The degree of sulfation in the liver is influenced by the hormonal status of the animal [2] and by cholestasis [3]. Sulfation of bile salts limits their reabsorption through the active transport system in the small intestine and, due to their polarity, through passive diffusion in the large intestine [4]. Sulfation of bile salts thus stimulates their fecal excretion.

It is well known that the intestinal microflora has a considerable influence upon the enterohepatic circulation and thus the fecal excretion of bile salts. Apart from this, the intestinal microflora also stimulates the rate of propulsion of the intestinal contents through the gastro-intestinal canal [5]. The first effect is due to specific biochemical action of the intestinal microflora upon the bile salts, e.g. deconjugation, dehydroxylation, desulfation and oxidation–reduction reactions, all of which may alter the efficiency of reabsorption of bile salts from the intestine and consequently alter the rate of excretion of these compounds. The second effect is probably due to non-specified effects of these bacteria and their metabolic products upon the gastro-intestinal wall of the host. These two phenomena have to be taken into account when comparing the rates of excretion of a particular bile salt in germfree (GF) and conventional (CV) rats. It has been shown that the intestinal microflora develops bile salt sulfatase activity [6–9] and thereby converts bile salt sulfates into less polar and more efficiently absorbed substrates [4, 10]. In this way, the desulfating microflora might interfere with the fecal excretion of bile salts and might influence the enterohepatic circulation of these compounds by improving the intestinal reabsorption.

Recently, a bile salt desulfating unnamed *Clostridium* species, termed strain S_1, was isolated from the rat intestinal microflora [11–13]. This strain deconjugates bile salts and desulfates the 3-sulfate esters of certain bile salts. In order to establish quantitatively the influence of this particular bacterial modification upon the rate of bile salt excretion, we compared the excretion of two bile salts, taurocholate (TCA) and taurolithocholate (TLCA), unsulfated and as 3-sulfated compounds, in GF rats, in CV rats and in gnotobiotic rats. Because of its strictly anaerobic character, *Clostridium* S_1 could not be established as a mono-associate in the digestive tract of gnotobiotic

[1]J.V.E. and S.H. are Research Assistants of the Belgian National Fund for Scientific Research (NFWO).

The following abbreviations have been used. GF = germfree; CV = conventional; GLC = gas–liquid chromatography; TLC = thin-layer chromatography; TLA = taurolithocholate; TLA-3-S = taurolithocholate-3-sulfate; TCA = taurocholate; TCA-3-S = taurocholate-3-sulfate.

rats. Association was, however, successful after previous association of GF rats with an unnamed *Clostridium* species termed strain Cl-8. This *Clostridium* Cl-8 is known to lower the oxidation–reduction potential of the intestinal contents and to deconjugate TCA for about 50%. Apart from this, the Cl-8 strain has little effect upon the metabolism of cholate and chenodeoxycholate. To exclude, however, any possible non-specific effect of *Clostridium* Cl-8 upon the gastro-intestinal function of the animals, a control group of gnotobiotic rats mono-associated with *Clostridium* Cl-8 was also studied.

EXPERIMENTAL

Animals and diet

Female inbred Fischer rats, approx 3 months old at the start of the experiments, were used. GF, gnotobiotic and CV animals were kept in Trexler's flexible-film plastic isolators (Standard Safety Equipment, Palatine, IL, U.S.A.) and were fed a steam-sterilized L-356-Rega diet [14] and water *ad libitum*. In each experiment, each group contained 5 rats, kept individually in separate cages. Cages were equipped with a double mesh-wire bottom and absorbing paper, to facilitate collection of feces and urine, and to prevent coprophagy.

One μCi of the labeled bile salt was injected intraperitoneally at time 0 h. Feces were collected at 24-h intervals during the first 4 days of the experiment and at 2-days intervals thereafter until sufficient excretion of label was attained. Urine collection was performed during the first week only, to check if any label was excreted via the urine. Cumulative excretion curves were obtained by transformation of the data as described by Lindstedt and Norman [15].

At the end of the experiments, the animals were killed to determine the cecal weight. The small intestine and the cecum plus the large intestine were homogenized and freeze-dried prior to determination of the intestinal bile salt pools.

Bile salts

Unlabeled lithocholic acid was obtained from Steraloids Inc., Wilton, NH, U.S.A. [24-^{14}C]Lithocholic acid was purchased from Amersham International (Amersham, England) and [24-^{14}C]taurocholate was obtained from NEN (Boston, MA, U.S.A.). The sulfur trioxide–triethylamine complex was prepared as described by Tserng and Klein[16]. Chlorosulfonic acid was obtained from Fluka AG (Buchs, Switzerland). Baker disposable extraction columns (Octadecyl; 6 ml) were purchased from J. T. Baker Chemical Co. (Phillipsburg, NJ, U.S.A.).

Preparation of [24-^{14}*C*] *taurolithocholate*. Radiolabeled lithocholic acid (1.027 mg; 150 μCi) was diluted with 75 mg unlabeled lithocholic acid, and dissolved in 4 ml 95% ethanol. To this solution were added 69.2 mg EEDQ, (*N*-ethoxy-carbonyl-2-ethoxy-1,2-dihydroquinoline) dissolved in 2.8 ml 95% ethanol, and 25 mg taurine dissolved in 0.4 ml 0.5 N NaOH. After boiling under reflux for 90 min, the components were distributed between ethyl acetate (72 ml) and water (10.8 ml) as described by Lack *et al.*[17]. The aqueous phase, containing the conjugated bile salts was evaporated to dryness, and the product was crystallized from hot ethanol. TLC in EBAW (ethyl acetate–*n*–butanol–acetic acid–water, 40:30:15:15, by vol), and scanning for radioactivity showed one peak.

Preparation of [24-^{14}*C*]*taurolithocholate*-3-*sulfate*. This compound was prepared according to Tserng and Klein[16]. Radiolabeled lithocholic acid (1.7 mg; 264 μCi), diluted with 125 mg unlabeled lithocholic acid, was dissolved in 1 ml dimethylformamide. After addition of 66.8 mg of sulfur trioxide–triethylamine, and stirring for 45 min at room temperature, EEDQ (115.56 mg), taurine (46.1 mg) and triethylamine (0.07 ml) were added and the solution was heated to 90°C with stirring for 30 min. Then, a second portion of 115.56 mg EEDQ was added, and the stirring was continued for 30 min at 90°C. After cooling, the reaction mixture was diluted with 1 ml of methylene chloride and filtered to remove unreacted taurine. One drop of water was added to the filtrate and the mixture was heated to 70°C for 30 min with stirring. After cooling, 16.6 ml of chilled diethyl ether were added while stirring, yielding a yellow oil. The stirring was continued in an ice-bath for 0.5 h. The ether-layer was removed by decantation and the oil was rinsed with ether (2 × 16.6 ml) in the same way. The triethylammonium salt was converted to the sodium salt by dissolving the oil in 4 ml of 0.2 N NaOH in methanol. The solution was filtered and rinsed twice with 0.5 ml of the same NaOH-solution. The filtrate was diluted with 8.4 ml of diethyl ether and, after storage at 5°C overnight, the precipitate was collected, washed with diethyl ether–methanol (10:3, v/v) and with diethyl ether. TLC in EBAW showed that the reaction product, besides TLCA-3-S (105.6 μCi), contained about 50 μCi of unsulfated TLCA. These two compounds were separated by column chromatography on Sephadex LH-20 with chloroform–methanol (1:1, v/v).

Preparation of [24-^{14}*C*]*taurocholate*-3-*sulfate*. Radiolabeled sodium taurocholate (0.517 mg; 50 μCi) was diluted with 25 mg unlabeled sodium taurocholate and dissolved in 1.5 ml of dry pyridine. The solution was cooled in an ice-bath and 0.1 ml of chlorosulfonic acid was added while stirring. After 1 min the reaction was interrupted by adding 1 ml of water and 3 ml of 1 N NaOH. The pyridine was removed by evaporation to dryness. The residue was dissolved in 10 ml of water and the pH of the solution was adjusted to 10. For desalting, this solution was percolated through a Baker octadecyl disposable extraction column. The column was rinsed with water until the effluent was neutral. Effluent plus washings were percolated through a second column, and the same procedure was repeated with a third and a

fourth column. The bile salts absorbed on each column were eluted with 10 ml of methanol. The methanol eluate from the first octadecyl column contained a small amount (3.88 μCi) of unreacted TCA. Pure TCA-3-S (31.23 μCi) was eluted from the three other columns with methanol, whereas the aqueous effluent contained small amounts of the disulfate and the trisulfate (5.3 μCi).

Determination of labeled compounds in feces and urine

Feces. Individual feces samples were collected in 50 ml conical Falcon tubes (Becton Dickinson & Co., U.S.A.) and homogenized with 10 ml of pre-cooled water. After freeze-drying, 20 ml of 80% ethanol were added and the tubes were kept in a water bath at 65°C for 4 h with regular shaking. After cooling, samples were centrifuged for 10 min at 4,000 *g*. Out of every tube, 3 samples of 0.5 ml were taken, and after adding 15 ml Lumagel (Lumac Systems Inc., Titusville, Fl, U.S.A.), counted twice for 5 min in a Packard Tri-Carb 2660 Liquid Scintillation System (United Technologies, Packard, Warrenville, IL, U.S.A.). To correct for quenching, quench-correction curves were stored in the memory of the Liquid Scintillation System and all results were consequently expressed as disintegrations per minute.

Urine. The absorptive papers were collected, dried in Erlenmeyer flasks and combined per cage. Fifty ml of 80% ethanol were added and the bile salts were extracted at 65°C for 4 h. Three samples of 1 ml extract plus 15 ml Lumagel were counted twice for 5 min. No rat was found to excrete in the urine more than 1% of the ^{14}C-label injected.

Thin-layer chromatography. TLC-analyses were carried out on silica gel G-plates (E. Merck, Darmstadt, W.-Germany) with EBAW for the sulfated or conjugated bile acids [18], and with IIAI i.e. isooctane–isopropanol–acetic acid–isopropylether (2:1:1:1, by vol) for the free bile acids [19]. After TLC, the labeled products were checked for purity by radioscanning (Dünnschicht Scanner II, Berthold, Germany). Radioscanning was also used to locate radioactive spots on TLC plates of fecal extracts.

Gas–liquid chromatography (GLC) of bile salts

To 500 mg of freeze-dried feces or intestine, 10 ml of 80% ethanol were added and this was kept at 65°C for 4 h. After sedimentation, 8 ml of the supernatant was desalted on an XAD-column (Servachrom-XAD, Serva, Heidelberg). Bile salts from GF rats and rats monoassociated with *Clostridium* Cl-8 were enzymatically deconjugated with cholylglycine hydrolase (Sigma Chemical Company, St Louis, U.S.A.) before separation of sulfated and non-sulfated bile salts. Fecal bile salts from CV rats and rats associated with *Clostridium* S_1 + *Clostridium* Cl-8 were shown to be completely deconjugated by the intestinal microflora and were not subjected to deconjugation prior to analysis. Sulfated bile salts were separated from non-sulfated bile salts on a Sephadex column (Sephadex LH-20, Pharmacia Fine Chemicals AB, Uppsala, Sweden) and the sulfated fractions were again desalted on an XAD-column. After solvolysis of total fractions and sulfated fractions, the bile salts were methylated and acetylated. A more detailed account of the method of analysis has been described elsewhere [20, 21]. To remove all traces of sterols, the preparation was chromatographed on a silica gel column (Kieselgel 60; 0.063–2 mm, E. Merck, Darmstadt, Germany). Sterols were first eluted with benzene and derivatized bile salts were then obtained by elution with acetone.

GLC of the methylester acetates was performed on a 3% OV-1 column at 268°C, on a 3% OV-17 column at 268°C and on a 1% QF-1 column at 240°C. The packing of columns was obtained from Applied Science Europe B.V., Oud-Beijerland, The Netherlands.

RESULTS

Radioscanning and GLC of fecal bile salts

To identify the major metabolites derived from the injected labeled TCA and TCA-3S the 80% ethanol extracts of fecal samples were subjected to TLC in EBAW and IIAI. Radioscanning of the plates showed that GF rats did not deconjugate nor desulfate bile salts. Rats monoassociated with *Clostridium* Cl-8 deconjugated bile salts for 50–75% but did not show any desulfating activity. In rats selectively associated with *Clostridium* S_1 plus *Clostridium* Cl-8, the labeled bile salts were completely deconjugated and desulfated. CV rats, however, transformed all cholate into deoxycholate and small amounts of an unidentified metabolite with the TLC mobility of lithocholate in IIAI.

The results of the GLC analysis of the total fecal bile salts of the gnotobiotic rats are shown in Table 1. Association with *Clostridium* Cl-8 or this Cl-8 plus *Clostridium* S_1 did not affect the total fecal bile salt output and had nearly no influence on the excretion or sulfation of allochenodeoxycholate-3-sulfate which is a predominating fecal bile salt in GF female rats [21]. In contrast, the 5β-bile salts were completely desulfated by *Clostridium* S_1. These results confirmed previous experiments *in vitro* showing that *Clostridium* S_1 desulfated and deconjugated the 3α-sulfates of the 5β-bile salts but did not desulfate the 3α-sulfates of allo-bile salts [12]. This strain S_1 also oxidized the 12-hydroxyl group of cholate to yield 12-ketochenodeoxycholate. Association with *Clostridium* Cl-8, or Cl-8 + S_1, did not affect the intestinal bile salt pools nor the cecal size (8–9% of body weight) of the gnotobiotic animals. The intestinal bile salt pools were 24.2 mg/100*g* body weight in gnotobiotic rats associated with *Clostridium* S_1 + *Clostridium* Cl-8 as compared to 25.7 mg in gnotobiotic rats monoassociated with *Clostridium* Cl-8 and 27.9 mg in GF rats. These pools were nearly twice as high as those of the CV rats (13.2 mg/100 g).

Table 1. Fecal excretion of bile salts by gnotobiotic female rats*

Bile salt	Percent of total bile salts		
	Germfree	+ *Clostridium* Cl-8	+ *Clostridium* Cl-8 + *Clostridium* S_1
Chenodeoxy	3.3 (26%)†	2.2 (33%)	2.5 (0%)
Allochenodeoxy	9.5 (85%)	11.5 (89%)	9.5 (70%)
Cholate + allocholate	46.2 (10%)	44.4 (12%)	26.3 (tr)
12-Ketochenodeoxy	0 (−)	0 (−)	18.5 (0%)
α-Muricholate	4.7 (35%)	2.2 (29%)	3.7 (0%)
β-Muricholate	34.9 (6%)	6.3 (26%)	5.7 (0%)
+ metabolite‡	0 (−)	26.4 (0%)	27.8 (0%)
Unidentified	±1.4 (?)	±7.0 (−)	±6.0 (−)
Total: mg/24 h/kg body weight	15.8	17.3	16.2
% Sulfated	17.3%	18.6%	6.6%
% Deconjugated	0%	55%	>90%

*12-Week old rats; 5 animals per group. Animals were fed purified diet L-356 (see ref. 14).
†In brackets: percent sulfated. tr = trace amounts, less than 1%.
‡Unidentified product formed from β-muricholate by *Clostridium* Cl-8 and presenting with a double bond in the side chain.

Table 2. Time (days*) required for 50% excretion of total label in the feces after intraperitoneal injection of ^{14}C-labeled bile salts

	TCA	TCA-3-S	TLCA	TLCA-3-S
GF rats	14.9	2.7	8.9	2.7
CV rats	6.6	3.1	4.4	1.5
Cl-8†	16.9	2.5	8	2.7
S_1 + Cl-8‡	13.3	12.2	12.5	11

*These values were calculated on best fit curves for $-\log(1-\mu t/a_0)$ transformations of cumulative excretion curves (see ref. 15).
†Gnotobiotic rats associated with *Clostridium* Cl-8.
‡Gnotobiotic rats associated with *Clostridium* S_1 + *Clostridium* Cl-8.

Excretion of labeled bile salts in GF and CV rats

The fecal excretion of unsulfated TCA and TLCA was faster in CV than in GF animals. This was demonstrated by the values of $t_{1/2}$ i.e. the time required for excretion of 50% of total label injected (Table 2) and by the cumulative excretion curves (Fig. 1). In GF rats the $t_{1/2}$ of labeled TCA and TLCA were 14.9 days and 8.9 days, respectively, as compared to 6.6 and 4.4 days in CV rats. It can also be seen (Table 2), that the $t_{1/2}$ values of labeled TLCA were consistently lower, in GF as well as in CV rats, than the $t_{1/2}$ values of TCA.

GF animals excreted TLCA-3-S ($t_{1/2} = 2.7$ days) more slowly than their CV counterparts ($t_{1/2} = 1.5$

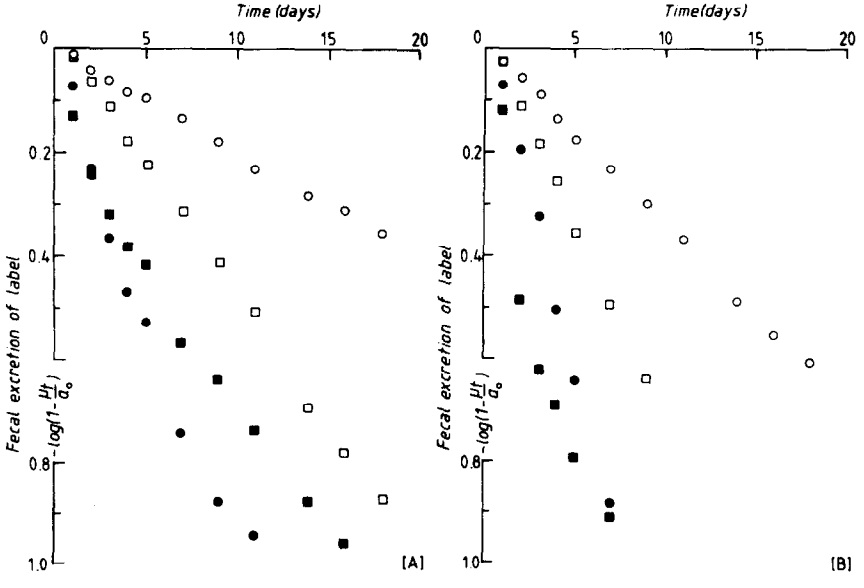

Fig. 1. Semilogarithmic plot of the cumulative excretion curves of taurocholate (Fig. 1A) and taurolithocholate (Fig. 1B) in GF rats (○) and CV rats (□) and of taurocholate-3-sulfate (Fig. 1A) and taurolithocholate-3-sulfate (Fig. 1B) in GF (●) and CV rats (■). For a discussion of this way of plotting, see Lindstedt and Norman (ref. 15); μ_t = total amount of label excreted at time t; α_0 = total amount of label injected. Fifty percent excretion of label is reached at $-\log(1-{}^{\mu}t/\alpha_0) = 0.301$.

days), as was the case for the unsulfated TLCA. For TCA-3-S, however, the situation was different: in CV rats the $t_{1/2}$ was 3.1 days, whereas in GF rats the $t_{1/2}$ was only 2.7 days. This is further illustrated by Fig. 1: after an initial phase (days 1 + 2) where there was nearly no difference between the excretion curves of TCA-3-S in GF and CV rats, the excretion by the GF rats was faster than that by the CV animals.

Both in GF and CV animals, the fecal excretion of sulfated bile salts was faster than that of the unsulfated compounds. As shown in Fig. 1, the cumulative excretion curves of TCA-3-S sloped down quicker than the respective curves of unsulfated TCA. In GF rats, $t_{1/2}$ of TCA-3-S was 2.7 days vs 14.9 for TCA and $t_{1/2}$ of TLCA-3-S was 2.7 days vs 8.9 for TLCA. The CV rats excreted 50% of the injected TCA-3-S in 3.1 days vs 6.6 days for TCA, and the $t_{1/2}$ of TLCA-3-S was 1.5 days vs 4.4 days for TLCA. Although there was no difference in $t_{1/2}$ times of TCA-3-S and TLCA-3-S in GF rats, in CV rats intraperitoneally injected TLCA-3-S ($t_{1/2} = 1.5$ days) was more quickly excreted via the feces than TCA-3-S ($t_{1/2} = 3.1$ days). This difference in fecal elimination by CV rats probably reflected intestinal desulfation followed by extensive resulfation of reabsorbed TLCA in rat liver [2, 9].

Effect of microbial desulfation upon fecal excretion of intraperitoneally injected labeled bile salt sulfates

To evaluate more specifically the influence of desulfation upon turnover and excretion time of TCA, TLCA and their sulfated analogues, we studied the excretion of these compounds in rats selectively associated with *Clostridium* strains S_1 + Cl-8 on the one hand, and strain Cl-8 alone as a control group on the other hand.

The 50% excretion values (Table 2) and the cumulative excretion curves (Figs 2 and 3) of the labeled bile salts in gnotobiotic rats monoassociated with *Clostridium* Cl-8, were similar to those of GF animals, indicating that *Clostridium* Cl-8 had no major influence on bile salt turnover and excretion rates.

In rats associated with *Clostridium* strains S_1 + Cl-8 the $t_{1/2}$ value of TLCA (12.5 days) was even higher than in GF rats (8.9 days) or in Cl-8 associated rats (8 days). Again, this probably reflected the hydroxylation and extensive sulfation of TLCA in rat liver [2, 9], coupled to the desulfating activity of strain S_1 in the intestine of the S_1 + Cl-8 asssociated rats. The combination of these effects would result in enhanced enterohepatic circulation of TLCA. In these rats the $t_{1/2}$ values of TCA-3-S and TLCA-3-S were of the same order as those of the unsulfated compounds and were 4–5 times higher than the $t_{1/2}$ values of TCA-3-S and TLCA-3-S in GF rats or in rats monoassociated with *Clostridium* Cl-8. The $t_{1/2}$ of TCA-3-S was 12.2 days in gnotobiotic S_1 + Cl-8 rats vs 2.7 in GF and 2.5 in Cl-8 rats. In the rats associated with *Clostridium* S_1 + Cl-8 the $t_{1/2}$ of TCA-3-S (12.2 days) was of the same order as that of TCA in GF rats (14.9 days) and the same remark applies to TLCA-3-S and TLCA. Similarly, the $t_{1/2}$ of TLCA-3-S was 11 days in gnotobiotic S_1 + Cl-8 rats vs no more than 2.7 days in GF and 2.7 in Cl-8 rats. The cumulative excretion curves of TLCA-3-S and TCA-3-S in *Clostridium* S_1 + Cl-8 associated rats were comparable to those of TLCA and TCA in GF rats. These data clearly demonstrate the importance of microbial desulfation upon the turnover and the enterohepatic circulation of bile salts.

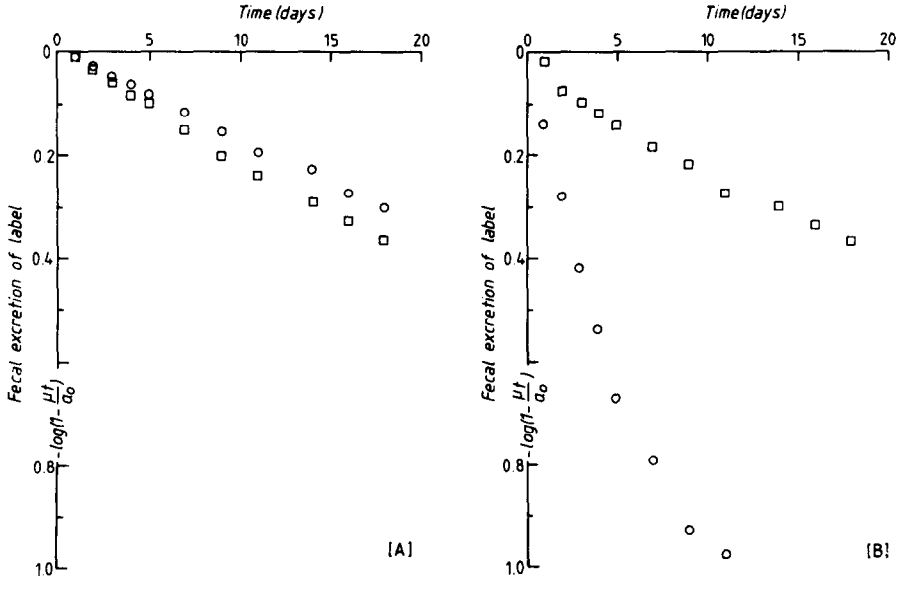

Fig. 2. Semilogarithmic plot of the cumulative excretion curves of taurocholate (Fig. 2A) and taurocholate-3-sulfate (Fig. 2B) in rats associated with *Clostridium* Cl-8 (○), or with *Clostridium* S_1 + *Clostridium* Cl-8 (□). Fifty percent excretion of label is reached at $-\log(1 - {}^{\mu}t/\alpha_o) = 0.301$. (See Fig. 1).

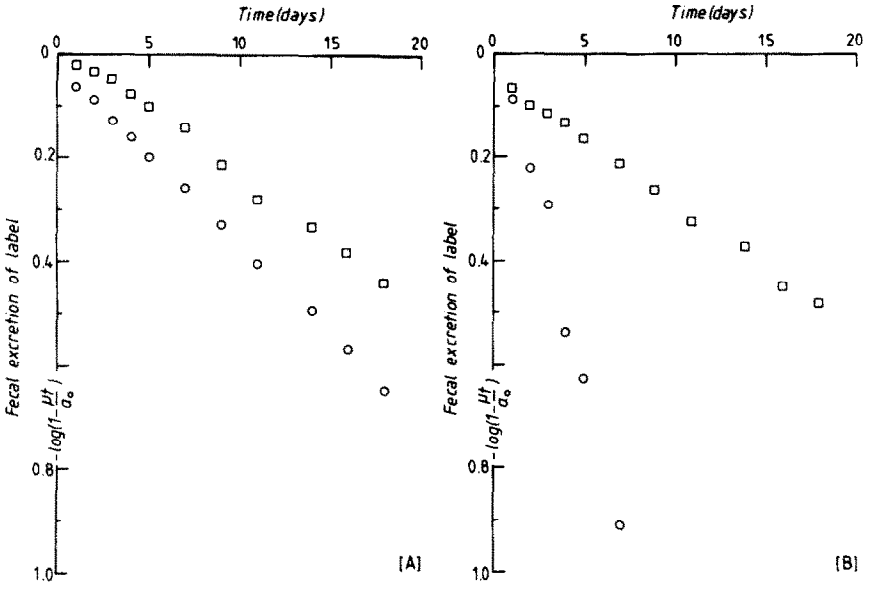

Fig. 3. Semilogarithmic plot of the cumulative excretion curves of taurolithocholate (Fig. 3A) and taurolithocholate-3-sulfate (Fig. 3B) in rats associated with *Clostridium* Cl-8 (○), or with *Clostridium* S_1 + *Clostridium* Cl-8 (□). Fifty percent excretion of label is reached at $-\log(1-{}^{\mu}t/\alpha_o) = 0.301$. (See Fig. 1).

DISCUSSION

Gustafsson *et al.*[22] established that the half life of cholic acid was 11.4 and 2 days in GF and CV rats, respectively. Since then, several reports have indicated that in the GF rat there is a more efficient reabsorption of bile salts from the intestinal tract, less fecal excretion of bile salts than in the CV rat [23], and in addition, a slower gastrointestinal transit [23, 24]. This results, at all levels of the intestine, in bile acid pools that are about twice the amount found in CV rats [25]. These findings are somehow related to the lower intestinal smooth muscle tone and the impairment of intestinal motility in GF rodents, the mechanism of which is insufficiently understood.

Our results are in accordance with previous findings [5] that the intestinal microflora stimulates the passage of the intestinal contents through the intestines and promotes fecal bile salt excretion. The time required for excretion of 50% of labeled unsulfated bile salts was consistently shorter in CV animals than in GF animals. In both groups, however, TCA-3-S and TLCA-3-S were much more rapidly excreted than their unsulfated analogues, again illustrating that sulfation impairs bile salt absorption.

In spite of the fact that CV rats have a smaller bile salt pool, a shorter intestinal transit time and increased bile salt turnover rates, the $t_{1/2}$ of TCA-3-S was still slightly shorter in GF than in CV animals. This was probably due to the desulfating activity of the intestinal microflora and reabsorption of the free bile salt in CV rats and testifies to the fact that desulfation of bile salts takes place in those intestinal segments from where partial reabsorption of the desulfated bile salts is still possible.

The pure effect of desulfation on the bile salt excretion rates could be studied by comparing the $t_{1/2}$ values of TCA-3-S and TLCA-3-S in gnotobiotic rats associated with *Clostridium* Cl-8 to the $t_{1/2}$ values of these compounds in rats associated with *Clostridium* S_1 + *Clostridium* Cl-8. These microorganisms deconjugated the bile salts, and strain S_1 also desulfated the bile salts, but they had no effect on the intestinal bile salt pools or on the cecal size of the animals. In these rats, the presence of the desulfating strain S_1 prolonged the 50% excretion times of TCA-3-S and TLCA-3-S by 350 and 300%, respectively, to values in the same range of time as those of unsulfated TCA and TLCA in GF animals and in animals monoassociated with *Clostridium* Cl-8. If the Cl-8 + S_1 microflora had had any major effect on the turnover and excretion of bile salts other than desulfation or deconjugation, then the cumulative excretion curves and $t_{1/2}$ values of TLCA-3-S and TCA-3-S in rats associated with *Clostridium* S_1 + Cl-8 would have differed greatly from those of TLCA and TCA in GF rats. This also confirms previous findings [26] showing that deconjugation has no major influence upon fecal excretion of bile salts.

In the gnotobiotic rats associated with *Clostridium* S_1 + *Clostridium* Cl-8, the $t_{1/2}$ of TCA-3-S was 4 times longer than in CV rats and in the same range of time as that of TCA in GF rats and in rats monoassociated with Cl-8. This shows that although extensive desulfation of bile salts takes place in CV rats, its effect on bile salt turnover is almost completely counteracted by other activities of the intestinal microflora, e.g. effects on the transit time, the cecal size or the bile salt pool. This however, did not prevent the TCA-3-S in the CV rats from being excreted more rapidly than its unsulfated analogue. The same remarks apply to the excretion values of TLCA-3-S.

In all groups of animals, the $t_{1/2}$ of TLCA was

shorter than that of TCA. This might be explained, at least partly, by the extensive sulfation of TLCA in rat liver [2, 9] and the limited reabsorption of TLCA-3-S from the intestine, which will tend to increase the fecal excretion of this toxic bile salt [9, 10]. The role of the hepatic sulfation of TLCA in TLCA metabolism is also illustrated by the fact that association of gnotobiotic rats with the desulfating strain S_1 prolonged the $t_{1/2}$ value of the labeled unsulfated TLCA by 40%.

All this points clearly to the importance of sulfation and desulfation upon bile salt excretion. Sulfation of bile salts clearly stimulated bile salt excretion; desulfation in gnotobiotic rats associated with *Clostridium* strains S_1 + Cl-8 clearly abolished this effect. We conclude that the effect of desulfation in CV animals is to lengthen the turnover of sulfated bile salts, but it must be remembered that $t_{1/2}$ values are in these circumstances the result of many influences, of which desulfation seems to be a major one.

The present data on the effects of sulfation and desulfation on the enterohepatic circulation of bile salts are in agreement with the results of previous studies on the effects of intestinal microbial steroid sulfatase activity on the metabolism of steroid hormones. Although estrone sulfate can be absorbed intact from the small intestine in GF rats, the non-sulfated product is absorbed much more efficiently, and absorption from the cecum is limited to the unsulfated compound [27]. The results of studies indicating that the steroid sulfatase and glucuronidase activities of the intestinal microflora promote the enterohepatic circulation of steroid hormones and their metabolites [28–31] are also in agreement with our findings.

Acknowledgements—This investigation was supported by a grant of the Belgian National Fund for Medical Research (NFGWO). The authors are indebted to Mr L. Verlooy and Mr R. Vandamme for skilful technical assistance in the experiments on gnotobiotic animals.

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