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DEFECTIVE BILE ACID TRANSPORT IN AN ANIMAL MODEL OF DEFECTIVE DEBRISOQUINE HYDROXYLATION

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Abstract—Bile acid transport in female DA (dark Aguti) rats, a model for debrisoquine hydroxylation deficiency in man, was investigated. Compared to hydroxylation competent male DA and Sprague–Dawley rats of either sex, the female DA rat had a significantly lower taurocholate maximal secretory rate *in vivo*. Studies in the perfused liver showed this to be due to a decreased extraction efficiency during exogenous taurocholate loading. To characterize further the defect, taurocholate uptake velocity into isolated hepatocytes was studied. This showed a decreased maximal uptake velocity in the female DA rat ($P < 0.02$). Whether this defect in bile acid uptake is related to the defective debrisoquine hydroxylation, remains to be established.

The female DA (DA for dark Aguti) rat has recently been described to have reduced capacity to hydroxylate debrisoquine as compared with other rat strains or its male counterpart [1]. Thus, it might serve as an animal model for the poor hydroxylation phenotype of the debrisoquine type in man [2, 3]. We have observed this rat to have higher random bile acid levels than Sprague–Dawley rats of either sex or male DA rats. We therefore investigated the possibility that the female DA rat might be spontaneously cholestatic and/or handle bile acids differently than Sprague–Dawley rats or its male strain mate.

MATERIALS

DA rats of either sex were obtained from the Hoffmann-LaRoche Center of Animal Supply (Füllinsdorf, Switzerland). Sprague–Dawley rats were purchased from the Süddeutsche Versuchstierfarm (Tuttlingen, Germany). All animals were acclimatized for at least 2 weeks prior to study. They were kept on standard rat chow and tap water *ad libitum* until the time of study. A twelve hour light–dark cycle was observed.

14C-taurocholate sodium (sp.A. 61 mCi/mmol) was obtained from New England Nuclear (Boston, MA). Taurocholate sodium was from Calbiochem Biochemical Corp. (La Jolla, CA). Both labeled and unlabeled taurocholate were >99% pure as assayed by thin-layer chromatography. Collagenase type II was purchased from Worthington Biochemical Co. (Freehold, NJ) and LumaGel from Lumac/3M (Scharberg, The Netherlands). Bovine serum albumin was fatty acid poor, >99% electrophoretically pure from Calbiochem. All other reagents were analytical grade from different commercial sources.

METHODS

Determination of the hydroxylation phenotype. Animals were given debrisoquine hemisulfate (5 mg/kg of the free base) by gavage in the evening and placed in metabolic cages. Urine was collected by 12 hours. Debrisoquine and 4-hydroxy-debrisoquine were determined by gas-liquid chromatography as described from this institution [4]. The ability to perform the benzylic hydroxylation was expressed as the metabolic ratio (debrisoquine/4-hydroxy-debrisoquine).

Taurocholate maximal secretory rate (SRm) was determined under pentobarbital anesthesia as previously described [5]. Briefly, the common bile duct and a jugular vein were cannulated with PE 10 and PE 50 tubing, respectively. Body temperature, controlled by a rectal thermometer, was maintained between 37.5 and 38.5° by a heating lamp. Taurocholate was infused at increasing rates (0.3–1.2 nmoles/100 g b.wtr). The three consecutive periods with the highest bile flow and bile salt secretion rates were averaged and taken as SRm. Four animals were studied in each group.

In situ *liver perfusion* was carried out as previously described [6, 7] using Krebs–Ringer-bicarbonate buffer containing 2% (w/v) bovine serum albumin, 0.1% (w/v) glucose and 20% (v/v) bovine erythrocytes. Taurocholate extraction was determined by injecting 0.1 μCi 14-C-taurocholate first in the absence of unlabeled taurocholate, then during a taurocholate infusion (0.3 μmoles/100 g b.wt.) which had been maintained for 30 min. This resulted in perfusate taurocholate concentrations of 15 ± 8 μmol/l, comparable to serum bile acid concentrations *in vivo*. The total hepatic venous effluent was collected for detection of radioactivity escaping

hepatic uptake. Bile was collected after injection of the 14C-taurocholate at 30-sec intervals for 30 min. Criteria of viability were as set forth before [7]; all reported experiments met these criteria. Five animals per group were studied.

Isolated hepatocytes were prepared as described before [8, 9] with the following modification: collagenase was used at a concentration of 0.8% (w/v) in calcium-free Krebs–Ringer bicarbonate buffer. The hepatocytes were kept on ice under a gentle flow of oxygen–CO_2 95:5. Prior to use, they were preincubated for 20 min at 37°. Time from preparation until use did not exceed 3 hr; viability as assessed by trypan blue exclusion did not change over this period. Taurocholate uptake velocity at 1–100 μM of taurocholate was determined as previously described by separating the hepatocytes from surrounding medium on Whatman GF/D glass fiber filters [8]. Trypan blue exclusion exceeded 85% in all the preparations. Hepatocyte preparations from 6 and 5 of the female and male animals in each group were studied, respectively.

Analysis of bile acids. [Carbonyl-14C]-taurocholate was added to 50 μl of rat bile for correction of losses. Solvolysis with sulphuric acid in ethyl acetate and alkaline hydrolysis were performed [10]. The liberated bile acids were extracted with Sep-Pak C18, passed through SP-Sephadex C25, isolated on Lipidex-DEAP and methylated with diazomethane. Coprostanol was added as internal standard and trimethylsilyl ethers were prepared and analysed by capillary gas-liquid chromatography–mass spectrometry using a Finnigan 1020 instrument equipped with a 20 m × 0.3 mm i.d. glass capillary column covered with barium carbonate and polyethyleneclycol 20'000 [11]. The following ions were used for quantification [12]: m/z 253 (cholic), m/z 255 (deoxycholic), m/z 285 (β-muricholic), m/z 370 (chenodeoxycholic, hyodeoxycholic acid, coprostanol), m/z 443 (α-muricholic acid).

Serum bile acids were quantitated by a commercially available radioimmunoassay kit (Beckton Dickinson). Total bile acids in bile were determined by the 3α-hydroxy steroid dehydrogenase method [13].

Data analysis. Taurocholate extraction efficiency was determined as E = (Dose-radioactivity recovered in hepatic venous outflow)/Dose and excretion as the cumulative excretion in bile up to 30 min after injection. Initial excretion velocity was expressed as the initial slope of the biliary excretion curve determined by multiexponential regression analysis. Retention was defined as the difference between extraction and cumulative excretion. Maximal taurocholate uptake velocity (V_{max}) and half-saturation constant (K_m) were determined by non-linear regression analysis of the Michaelis–Menten equation [14]. All results are expressed as mean ± 1 standard deviation. The means of the four groups were compared by a modification of Peritz' F-test [15]. $P < 0.05$ was considered statistically significant.

RESULTS

All female DA rats (N = 15) metabolized debrisoquine inefficiently, their metabolic ratio of debrisoquine/4-OH-debrisoquine averaging 1.01 ± 0.44 (range 0.40–1.83). The metabolic ratios in male DA (N = 14), and male (N = 14) and female (N = 15) Sprague rats were 0.13 ± 0.03, 0.15 ± 0.07 and 0.2 ± 0.10, respectively. The metabolic ratio of female DA rats showed no overlap with any of the three other groups and thus was statistically significant at $P < 0.001$ against each of the other groups. No statistical differences were noted between any of the control groups.

Serum bile acid, taken at random, were significantly ($P < 0.005$) higher in female DA rats (17.3 ± 9.4 μmol/l; N = 10) than in female SD rats (4.8 ± 1.5 μmol/l; N = 10). By contrast, basal biliary bile salt output showed no sex or strain difference, averaging 63 ± 18, 47 ± 11, 54 ± 11 and 46 ± 12 nmoles min^{-1} g liver in female DA, male DA, female SD and male SD rats, respectively. Relative biliary bile acid composition, determined by capillary gas-liquid chromatography/mass spectrometry was determined in each female DA and SD rats. The results are given in Table 1. Female DA rats had a significantly lower relative content of cholic acid in bile, while α- and β-muricholate were increased.

Table 1. Biliary bile acid composition (mol-%)

	Female SD rat	Female DA rat
Cholic acid (3α,7α,12α-trihydroxy-5β-cholanoic acid)	65.3 ± 9.7	47.2 ± 9.7*
Chenodoexycholic acid (3α,7α-dihydroxy-5β-cholanoic acid)	8.2 ± 6.4	9.7 ± 4.3
α-Muricholic acid (3α,6β,7α-trihydroxy-5β-cholanoic acid)	9.9 ± 2.6	23.9 ± 7.2†
β-Muricholic acid (3α,6β,7β-trihydroxy-5β-cholanoic acid)	3.0 ± 1.5	9.3 ± 2.8†
Deoxycholic acid (3,12α-dihydroxy-5β-cholanoic acid)	7.3 ± 8.2	4.6 ± 3.0
Hyodeoxycholic acid (3α,6α-dihydroxy-5β-cholanoic acid)	6.3 ± 3.8	5.5 ± 3.5

* $P < 0.005$.
† $P < 0.001$.

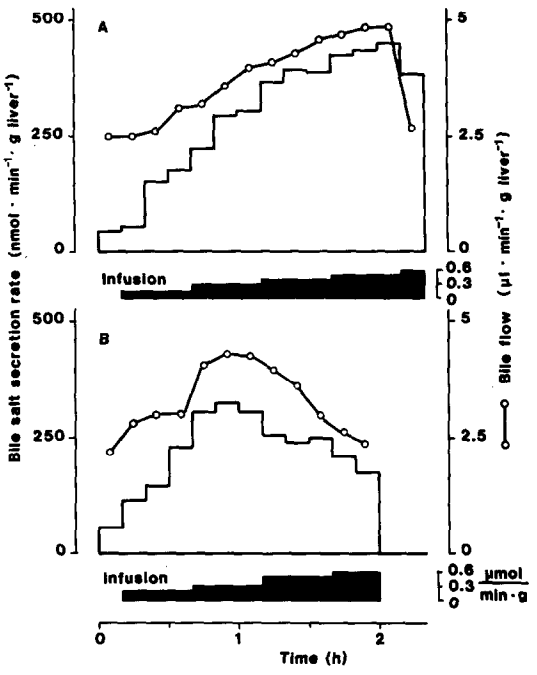

Fig. 1. Determination of taurocholate SRm in a female SD (A) and DA (B) rat.

Taurocholate maximal secretory rate (SRm) was determined *in vivo*. A typical experiment in a female DA and SD rat is shown in Fig. 1. Taurocholate maximal secretory rate averaged 346 ± 42 nmoles/min/g liver in female DA rats; this was significantly less than in male DA rats (429 ± 58; $P < 0.05$) and female and male Sprague–Dawley rats, in which SRm averaged 429 ± 10 ($P < 0.05$) and 447 ± 25 ($P < 0.05$) nmoles·min^{-1}·g^{-1} liver, respectively. None of the differences between the other groups were statistically significant. Spontaneous bile flow and bile flow during SRm are given in Table 2. While there was no statistically significant difference between spontaneous bile flow between the different groups, bile flow during SRm was significantly lower in the female DA rat as compared to the three control groups.

In situ rat liver perfusion was carried out to characterize single pass taurocholate extraction. No differences in viability criteria, such as spontaneous bile flow (Table 2), transaminase or potassium release (data not shown), were apparent between the different groups. Single pass taurocholate extraction in the absence and presence of a taurocholate load are reported in Table 3. It was similar in all four groups in the absence of exogenous bile acids. By contrast, a significantly lower extraction was observed when the transport system was stressed by a 0.3 µmol/100 g taurocholate infusion (Table 3). Cumulative taurocholate excretion from 0 to 25 min after injection of labeled taurocholate is also reported in Table 3. Similar results as with taurocholate extraction were observed, i.e. female rats excreted a tracer dose of taurocholate as efficiently as the three control groups, while cumulative taurocholate excretion during a 0.3 µmol/100 g b.wt. taurocholate infusion was significantly less in the female DA rat.

Taurocholate excretion rate into bile could adequately be described by a biexponential function (Table 4, Fig. 2). The fast component (α) did not show any differences in the absence or presence of taurocholate. The slow component (β) was equal in the four groups in the absence of unlabeled taurocholate, but was significantly lower in the female DA rat as compared to either sex SD rats (Table 4) during infusion of taurocholate.

Hepatocyte yield and viability were comparable in the four groups. Yield averaged 1.3 ± 0.2, 1.8 ± 0,5, 1.8 ± 0.8 and 2.4 ± 0.7 × 10^{+8} cells/liver in the female DA, male DA, female SD and male SD rat, respectively. The corresponding values for trypan blue staining were 12 ± 3, 8 ± 3, 15 ± 6 and 7 ± 2%, respectively. None of these differences reached statistical significance.

Taurocholate uptake into isolated hepatocytes of all four groups of rats could adequately be described by the Michaelis–Menten equation. The results in the female and Sprague–Dawley rats are shown in Fig. 3. Each experiment was fitted individually to the Michaelis–Menten equation. Statistical tests were then carried out using the parameters calculated from each individual experiment and are reported in Table 5. The half saturation constant K_m was comparable in the four groups of rats, while the maximal uptake velocity V_{max} was significantly lower

Table 2. Bile flow (µl : min^{-1} : g liver^{-1}) in different rat strains *in vivo* and in the perfused liver ($\bar{x}$ ± S.D.; in each group)

Strain (N per group)	*in vivo* Basal	*in vivo* during SRm* (4)	Perfused liver BSIF†	Perfused liver BSDF‡ (5)
Female DA	2.60 ± 0.36	4.57 ± 0.254§	1.33 ± 0.36	2.51 ± 0.30
Male DA	2.36 ± 0.20	5.11 ± 0.16	1.37 ± 0.09	2.40 ± 0.24
Female SD	2.64 ± 0.25	5.10 ± 0.19	1.30 ± 0.42	2.60 ± 0.20
Male SD	2.40 ± 0.12	5.39 ± 0.26	1.32 ± 0.23	2.22 ± 0.27

* Maximal secretory rate of taurocholate.
† After bile salt depletion.
‡ During taurocholate infusion (0.3 µmol : min^{-1} : 100 g body weight^{-1}).
§ Significantly different from DA male ($P < 0.02$), SD female ($P < 0.03$) and SD male ($P < 0.005$).

Table 3. Taurocholate extraction efficiency and cumulative biliary excretion (0–30 min) in the perfused liver of different rat strains and sexes ($\bar{x} \pm$ S.D., N = 5 per group)

Taurocholate dose:	Trace		0.3 μmol/min	
Strain	Extraction	Excretion	Extraction	Excretion
Female DA	0.98 ± 0.02	0.78 ± 0.16	0.68 ± 0.16*	0.47 ± 0.27*
Male DA	0.98 ± 0.01	0.99 ± 0.07	0.94 ± 0.04	0.93 ± 0.11
Female SD	0.97 ± 0.01	0.85 ± 0.13	0.93 ± 0.03	0.86 ± 0.11
Male SD	0.97 ± 0.01	0.88 ± 0.07	0.94 ± 0.03	0.96 ± 0.02

* Significantly different from male DA, female SD and male SD (P at least < 0.05 by Peritz' F-test).

Table 4. Taurocholate excretion rate constants into bile in rats of different strains and sexes ($\bar{x} \pm$ S.D., N = 5 per group)

	Trace		0.3 μmol/min	
Taurocholate dose	α	β	α	β
Female DA	0.44 ± 0.15	0.08 ± 0.05	0.40 ± 0.20	0.04 ± 0.03[a]
Male DA	0.71 ± 0.38	0.14 ± 0.04	0.55 ± 0.06	0.11 ± 0.06
Female SD	0.54 ± 0.18	0.12 ± 0.02	0.38 ± 0.05	0.11 ± 0.05
Male SD	0.46 ± 0.18	0.10 ± 0.05	0.53 ± 0.10	0.11 ± 0.06

[a] P < 0.05 against male and female SD rats.

in the female DA rat as compared to all three control groups (Table 5).

DISCUSSION

The female DA rat has recently been established as an animal model for hydroxylation deficiency of the debrisoquine type [1]. This rat strain has also been shown to have elevated cholesterol levels [16]. We observed increased random serum bile acid levels in the female DA rats. Our present investigations demonstrate an uptake defect for bile acids characterized by a decreased maximal uptake velocity in isolated hepatocytes; this defect can be made apparent in the perfused liver when the bile acid transport system is stressed by an exogenous bile acid infusion corresponding to slightly higher bile acid load than that occurring in response to a meal. *In vivo*, maximal bile acid secretory rate is lower in the female DA rat as compared to all three control groups. The main qualitative difference in biliary bile acids was a decrease in cholate and an increase in muricholate content in female DA rats.

Table 5. Kinetic parameters of taurocholate uptake into hepatocytes isolated from rats of different strain and sexes ($\bar{x} \pm$ S.D.)

Strain	(N)	K_m (μmol/l)	V_{max} (nmol : min^{-1} : 10^6 $cells^{-1}$)
Female DA	(6)	22 ± 14	0.85 ± 0.30*
Male DA	(5)	49 ± 36	1.54 ± 0.50
Female SD	(6)	22 ± 11	2.15 ± 0.34
Male SD	(5)	38 ± 14	1.58 ± 0.39

* P at least <0.02 against the three other groups by Peritz' F-test.

To exclude that any difference found could be due to either a difference in strains or sex, three control groups had to be included. Thus, the finding that the female DA rat differs in different aspects of bile acid handling both compared to its male strain counterpart, the male DA rat, as well as compared to a female rat of another, the Sprague–Dawley strain, supports the contention that the differences reported are not due to either strain or sex differences. The latter point is further confirmed by the finding that there was no difference between female and male Sprague–Dawley rats in any of the parameters tested.

It is quite unlikely, that any of the observed differences are due to increased susceptibility of the female DA rat liver to the procedures used. First, experiments *in vivo*, in the perfused liver and in isolated hepatocytes gave internally compatible results. Second, none of the different parameters of viability tested showed compromise of the perfused liver or of the isolated liver cells.

The main mechanism for the altered bile acid handling seems to be the impaired maximal uptake velocity in isolated hepatocytes. This is confirmed by the finding of a decreased extraction efficiency only when the taurocholate transport system is stressed by an infusion of exogenous bile acid but not when a tracer dose of taurocholate is used. Somewhat more difficult to understand is the decreased maximal secretory rate *in vivo*. SRm has been thought to probe the excretory transport maximum [17]. It has been demonstrated quite clearly, however, that SRm assessed by the infusion method represents the balance between toxicity of the bile acid studied and its transport capacity into bile [5, 18]. Comparison of maximal secretory rate and maximal uptake velocity in the perfused rat liver had suggested that the latter is about six times higher than the excretory step [6]. Maximal uptake velocity in these studies was

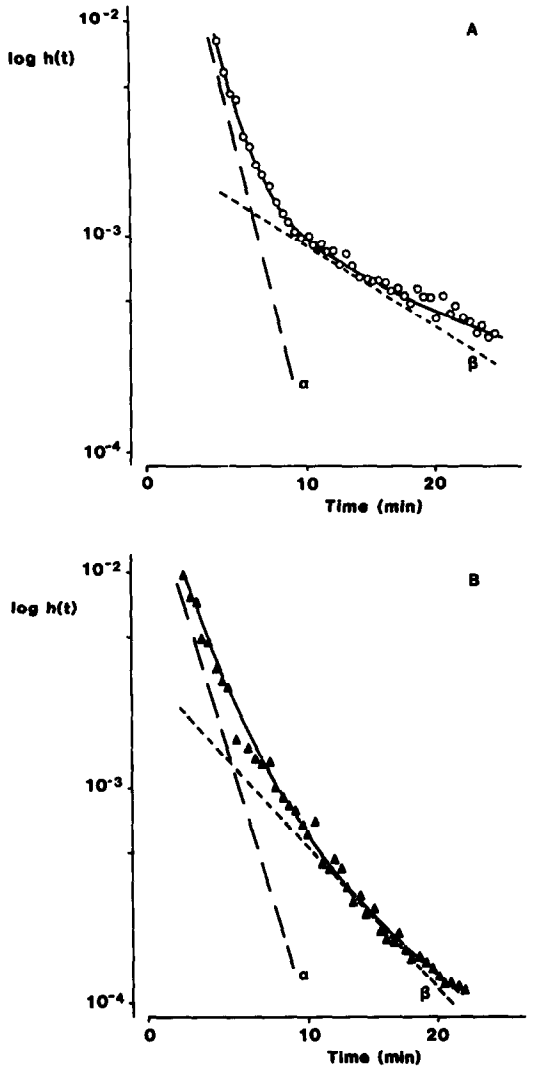

Fig. 2. Biliary ^{14}C-taurocholate secretion rate during a 0.3 μmol/min taurocholate infusion in a female DA (A) and male Sprague–Dawley (B) rat. The ^{14}C-taurocholate in bile is expressed as the fraction of the dose (h(t)) recovered in bile in each 30-sec sample. The smooth(−) line gives the theoretical fit; the individual rate constants are indicated as (− − −) and (− · − · −) for the α and β phase, respectively. The equations describing taurocholate secretion were

$y(t) = 0.445.e^{-0.651.t} + 0.021.e^{-0.079.t}$ and

$y(t) = 0.311.e^{-0.494.t} + 0.036.e^{-653.t}$ for A and B, respectively.

determined by single bolus injections of the bile acids; a preliminary report using constant infusion suggested, however, that the capacity of the two processes is about equal [19]. Thus, it is conceivable that SRm *in vivo* is determined by maximal uptake velocity rather than by the secretory step. A precedent for this notion is ethinyl estradiol-induced cholestasis. Thus, decreased SRm has been described after estrogen treatment [20] which was recently found to be associated with decreased hepatocellular uptake [9]. Our data do not permit to determine whether uptake is rate-limiting for SRm *in vivo*.

There was no difference in spontaneous bile acid secretion rate between the four groups. Female DA rats had higher random serum bile acid levels than female SD rats. There was no relationship between serum bile acid levels and biliary bile acid excretion. This is not surprising since portal rather than peripheral serum bile acid concentration is thought to determine biliary secretion [21]. Portal bile acid concentrations were not studied in the present investigation. The elevated peripheral serum bile acid concentrations reflect spillover due to the uptake defect [21]. Whether the altered bile acid composition contributes to the elevated serum bile acid concentrations cannot be judged from the present data since the hepatic extraction efficiency of muricholate is unknown.

Two additional differences in bile acid handling between the DA rat and the controls deserve comment: (1) a higher 25 min hepatic retention of bile acids during a taurocholate load (difference between hepatic extraction and cumulative biliary excretion; see Table 3) and (2) a reduced slow secretory component (β; see Table 4) observed again during a bile acid load. Neither defect alone appears large enough to explain the decreased SRm observed *in vivo* and it is quite likely that they are due to an alteration of the secretory characteristics of centrizonal hepatocytes. Thus, Groothuis *et al.* demonstrated that centrizonal hepatocytes take up bile acids equally well as periportal hepatocytes. Centrizonal hepatocytes, however, were considerably less efficient at excretion [22]. We have shown in preliminary experiments that the β-phase during normal, antegrade perfusion corresponds to the secretory rate obtained during retrograde perfusion [23]. Retrograde perfusion is thought to probe centrizonal hepatocytes [22]. It is thus conceivable that pericentral hepatocytes of female DA rats have secretory characteristics different from periportal hepatocytes. An alternative explanation could be a difference in quantity or affinity of the recently described cytosolic bile acid binding proteins [24].

Thus, a decreased maximal uptake velocity seems to be the main defect explaining the different bile acid handling by female DA rats. A decreased maximal uptake velocity could theoretically be due to altered number of bile acid carriers on the plasma membrane [25], an alteration in the driving forces [6, 9] or alterations in the characteristics of the sinusoidal plasma membrane [26]. Future investigations will have to elucidate which one of these mechanisms is altered in the female DA rat.

Female DA rat bile contained less cholic acid than the bile of female SD rats (Table 1); this was compensated for by an increase in the content of α-muricholate and its intestinal conversion product, β-muricholate. Whether 12-α-hydroxylation, a cytochrome P-450-dependent step in cholate biogenesis [27], is decreased in the DA rat and/or whether 6-α-hydroxylation, a cytochrome P-450-dependent step in α-muricholate synthesis [28] is induced in the DA rat cannot be answered from our studies. Both hydroxylation pathways are interdependent, depend on hormonal and microbiological environment

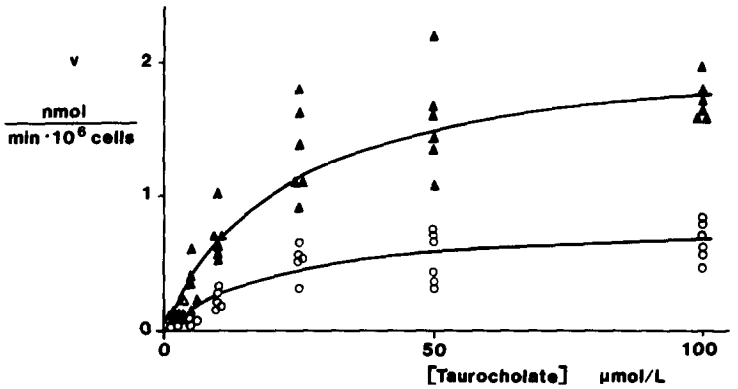

Fig. 3. Taurocholate uptake velocity (v) in female Sprague–Dawley (▲) and DA (○) rats. Uptake velocity as a function of the taurocholate concentration (S) could adequately be described by the Michaelis–Menten equation. The equation was $v = 2.15.S/(22 + S)$ and $v = 0.85.S/(22 + S)$ in the female Sprague–Dawley and DA rat, respectively.

[12, 27] and the difference in bile acid composition, therefore, is not necessarily related to the debrisoquine hydroxylation deficiency.

At present it is unclear whether deficient debrisoquine hydroxylation and impaired bile acid uptake in the female DA rat are related. The former is a function of the smooth endoplasmic reticulum and the latter one of the basolateral membrane. A generalized alteration in membrane lipid composition or the presence of an endogenous inhibitor of both processes could perhaps explain both defects. However, recently a debrisoquine hydroxylase has been partially purified from rat liver microsomes; this enzyme could not be demonstrated in the female DA rat [29]. This finding seems to favor a single protein defect rather than a generalized membrane defect or presence of an endogenous inhibitor.

The observation by Nadir *et al.* that hypercholesterolemia was linked to hydroxylation deficiency led them to postulate defective bile acid biosynthesis in the female DA rat [16]. Our own data could lead to another speculation, namely that the bile acid transport defect leads to hypercholesterolemia by deficient feed-back inhibition of cholesterol hydroxylation to bile acids. Such a feed-back inhibition has been demonstrated directly in the bile fistula rat [30] but may be mediated by some unknown intermediate [31]. At present, there is no experimental support for the hypothesis that bile acid hydroxylation provides the common link between the defect in bile acid transport and drug hydroxylation; in particular, no differences between urinary bile acid and bile alcohols were found in a small study on human deficient debrisoquine hydroxylators [32].

In conclusion, our studies have demonstrated impaired bile acid handling by the female DA rat, an animal with known defective debrisoquine hydroxylation. Compared with three control groups, this defect was found in the female DA rat only and thus did not represent a sex or strain difference. The defect seems to involve mainly a decreased capacity of bile acid uptake at the level of the basolateral membrane, although an additional defect at the level of intracellular storage or secretory characteristics of centrizonal hepatocytes could contribute to impaired bile acid handling. Future studies will have to determine whether this represents a generalized membrane defect in the female DA rat or whether the two findings are unrelated.

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