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Gender-Differential Liver Plasma Membrane Affinities in Hepatic Tetrabromosulfonephthalein (TBS) Uptake

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ABSTRACT. The sex difference in the hepatic uptake of tetrabromosulfonephthalein (TBS) was investigated in male and female rats in two different experimental models. In the intact animal, the initial plasma disappearance constant rate, the initial velocity of uptake, and the plasma clearance of TBS were significantly higher in females than in males. In sinusoidal liver plasma membrane vesicles, kinetic parameters of TBS uptake were investigated in both sexes. The K_m was lower in females than in males (5.5 ± 0.4 vs 17 ± 4 μM, $P < 0.05$), whereas V_{max} showed comparable values (544 ± 15 vs 581 ± 60 nmol TBS/min/mg protein, mean ± SD, NS, females and males, respectively). Collectively, these data indicate that the sex difference in hepatic uptake of TBS is located at the sinusoidal liver plasma membrane and is due to a greater affinity of the electrogenic transport system(s) in females. BIOCHEM PHARMACOL 51;9:1117–1122, 1996.

KEY WORDS. organic anions; liver uptake; sex difference; liver plasma membrane vesicles

Sex differences have been described for a variety of liver functions, including enzymatic activities, reactions to drugs, and transport processes. Many endogenous and exogenous compounds, such as bilirubin, fatty acids, BSP†-glutathione and indocyanine green, have been shown to be transported more rapidly by the female liver [1–4]. This finding agrees with other observations in human physiology and disease. It has been reported that the serum bilirubin level is lower in adult females [5] and that Gilbert's syndrome, a pathological condition characterized by unconjugated hyperbilirubinemia and impaired hepatic transport of organic anions, is much more frequent in males than in females [6, 7].

In the intact rat, it has been reported that females show a faster and more efficient uptake of BSP, a cholephilic dye employed in diagnostics for an overall assessment of liver function [8], than do males [9]. The same difference is seen in the perfused liver [9]. In isolated hepatocytes, the K_m value of the high affinity BSP uptake has been found to be lower in female than in male rats [9]. A closer insight into the problem has shown that this sex-related difference is controlled by estrogens [10]. All these studies were done in complex experimental models and did not clarify at what specific cellular level this difference was located.

Several independent investigators have provided evidence that organic anions cross the liver plasma membrane by carrier-mediated mechanisms [11–21]. Some of these studies have pointed to a 37 kDa protein, bilitranslocase, as the putative organic anion transporter [11–13]. This protein accounts, at least in part, for the electrogenic transport of organic anions across the sinusoidal liver plasma membrane [13, 21, 22].

Organic anions such as BSP, thymol blue, and TBS have been reported to be translocated via bilitranslocase. These dyes have a visible spectrum and pH indicator properties. Taking advantage of these characteristics, thymol blue, BSP, and TBS transport in liver plasma membrane vesicles has been studied with the use of a spectrophotometric technique [13, 22, 23]. The physicochemical characteristics of TBS [22] render this anion the more suitable for the study of liver uptake in plasma membrane vesicles employing the spectrophotometric technique previously indicated.

The aim of the present study was to determine if the initial plasma disappearance rate of TBS is higher in females than in males and, in this case, to test the hypothesis that the primary event mediating such a difference resides at the level of the plasma membrane domain.

MATERIALS AND METHODS

Female and male Wistar rats (60–90 days old) weighing 200–250 g were used throughout the study. Animals were allowed free access to a standard laboratory chow and tap water, and were housed in a constant temperature–humidity environment.

In Vivo *Clearance Studies*

The initial plasma disappearance rate and plasma clearance of TBS were measured according to Orzes *et al.* [9]. Briefly,

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† *Abbreviations:* BSP, sulfobromophthalein; TBS, tetrabromosulfonephthalein; *k*, initial plasma disappearance constant rate; V, initial velocity of uptake; Vd, plasma distribution volume; and Cl, plasma clearance.

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the animals were anesthetized with sodium pentobarbital (50 mg/kg body wt). Body temperature was monitored continuously with a deep rectal probe and maintained at 37 to 37.5° with an infrared lamp. The femoral vein and artery were cannulated with PE-50 tubing. TBS, 1.5 μmol/kg body wt, dissolved in saline solution in a final volume of 0.1 mL/100 g was injected i.v. as a bolus. Blood samples were taken from the femoral artery at 15-sec intervals for 120 sec and were collected into 1.5-mL polyethylene tubes containing 5 U heparin. Samples were spun at 1000 *g* for 4 min in an Eppendorf centrifuge, and the dye concentration was assessed spectrophotometrically after alkalinization. Data were plotted on a semi-logarithmic scale, and the initial plasma disappearance constant rate (k, min^{-1}), which mainly reflects hepatic uptake, was calculated by a computer program (Enzfitter). The initial velocity of uptake (μmol/min/kg) and plasma clearance (mL/min/kg) were determined as described [9, 24]. The primary distribution volume of the dye (mL/kg) was calculated from the ratio dose/C_0, where C_0 is the extrapolated plasma concentration at 0 time.

Measurement of Transport Activity in Liver Plasma Membrane Vesicles

Liver plasma membrane vesicles were prepared as previously described [21, 22] in a medium containing 10 mM HEPES, 250 mM sucrose, pH 7.4, and stored in liquid nitrogen until used (within 4 weeks). The degree of purity was determined by measuring the activity of Na^+,K^+-ATPase [25], 5′-nucleotidase [26], glucose-6-phosphatase, and succinate-cytochrome *c* reductase [27]. The release of inorganic phosphate was measured according to Widnell [28]. Protein concentration was assessed by the bicinchoninic acid protein assay according to Smith *et al.* [29]. The relative specific activities (RSA) of Na^+,K^+-ATPase and 5′-nucleotidase were enriched 17 and 3 times, respectively, over the starting homogenate, while contamination with either endoplasmic reticulum (RSA of glucose-6-phosphatase 0.25) or mitochondria (RSA of cytochrome *c* reductase 0.16) was low. This result indicates that the plasma membrane vesicles were derived mainly from the basolateral domain of the hepatocytes. No differences between sexes were observed in the specific activities of the marker enzymes.

TBS movements in vesicles were followed spectrophotometrically by a dual wavelength recording spectrophotometer at room temperature, as previously described for TBS and BSP [13, 22, 23]. This assay has been used to measure the movement of phthaleins in proteoliposomes [11], erythrocyte ghost vesicles [30], isolated hepatocytes [31], and isolated plasma membrane vesicles [22, 23, 32–37]. In detail, phthalein dyes are known to be pH indicators, i.e. their spectral properties change as a function of proton concentration. Plasma membrane vesicles may be prepared so that the pH inside is close to physiological pH. When vesicles are added to a solution of a phthalein dye at pH 8.1, any entry of the pH indicator into the less alkaline inner space is followed by a decrease in absorbance at the maximal wavelength of the deprotonated form of the dye [30, 32]. This effect can be used to follow the kinetics of entry of the dye into the inner compartment. The use of dual wavelength spectrophotometry minimizes light scattering in the system. Various optical artifacts, the most relevant of which is the optical change induced by absorption of the dye onto the surface of the membrane, interfere with the measurements. This phenomenon is rapid and is distinguished easily from the transport process [31]. The sample is pre-equilibrated with the phthalein in the presence of a high concentration of potassium. Subsequently, valinomycin is added, and the diffusion potential created may drive further entry of the dye. It was shown that the initial rate of this process represents a quantitative measure of the activity of the carrier involved [32]. In the present work, vesicles were added to a TBS solution (0–90 μM) at pH 8.1. Any entry of the pH indicator into a less alkaline inner space is followed by a decrease in absorbance at the λ_{max} of the deprotonated form of TBS. Uptake determination was started by the addition of 25 μL vesicles (60 μg protein) to 1.975 mL medium containing 0.1 M potassium phosphate buffer, pH 8.1. After 15 sec, 6 μL of valinomycin (0.5 μg/μL in methanol) was added to the cuvette, by threading the needle of a calibrated glass syringe through a hole in the magnetically stirred cell of the spectrophotometer. The initial uptake rate (nmol TBS · sec^{-1} · mg $protein^{-1}$) was calculated from the linear portion of the curve that follows the addition of valinomycin as previously described [22].

All determinations were performed in triplicate, and all

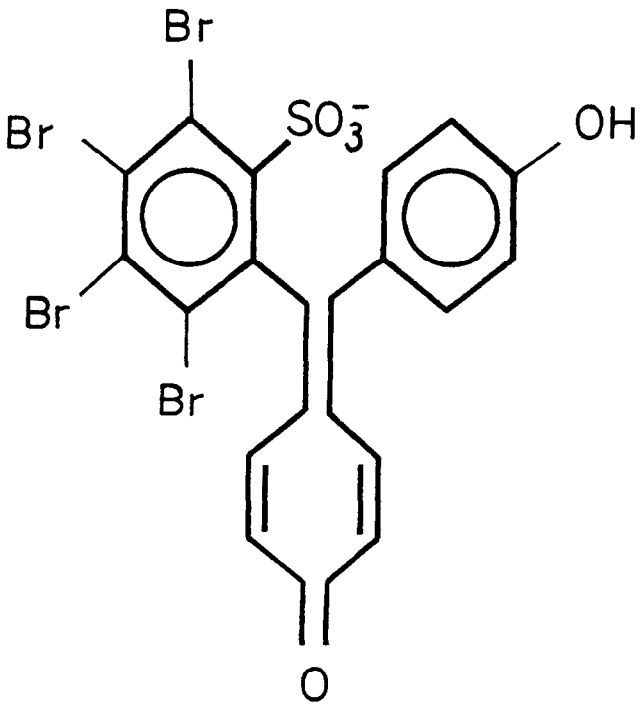

FIG. 1. Structural formula of tetrabromosulfonephthalein (TBS) in the deprotonated colored quinoid form of the dye.

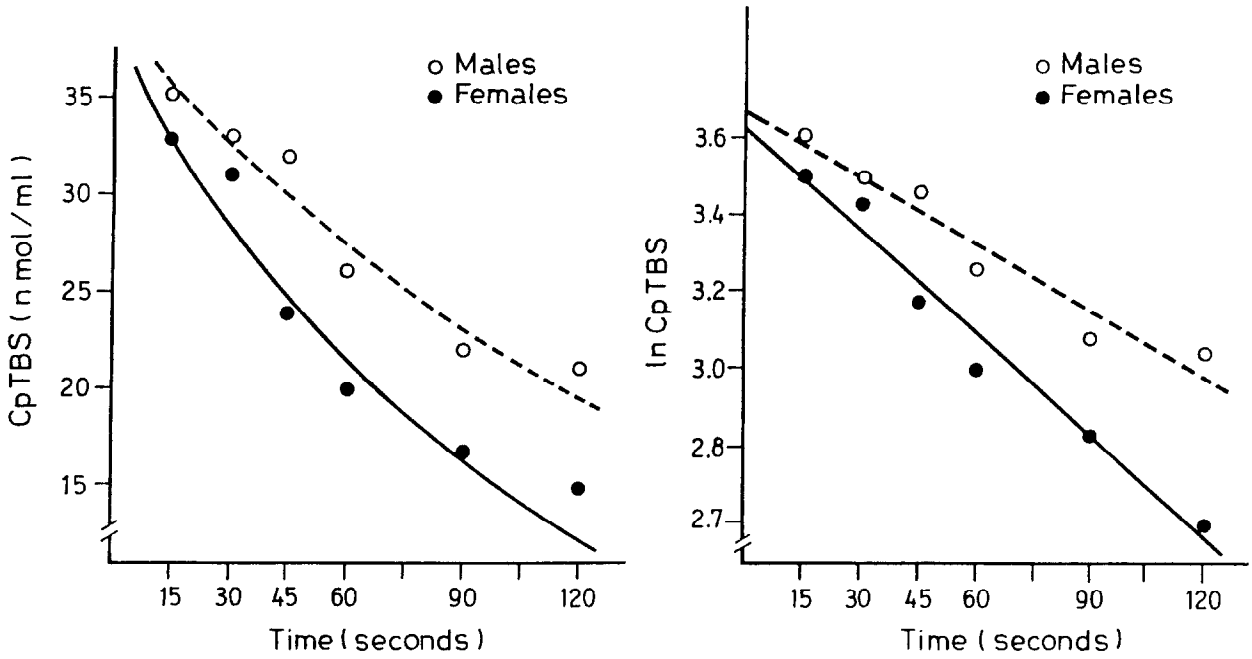

FIG. 2. Plasma TBS concentration vs time curve after administration of an i.v. bolus of 1.5 μmol/kg body weight to male and female rats. The same data are plotted on a semi-logarithmic scale in the right-hand panel. Data obtained from a typical experiment are represented.

observations were confirmed with two or more separate membrane preparations.

Materials

Chemicals were purchased from the Sigma Chemical Co. (St. Louis, MO, U.S.A.) and were analytical grade pure. TBS was obtained from the Aldrich Chemical Co. (Milwaukee, WI, U.S.A.).

Statistical Analysis

All results are expressed as means ± SD. The significance of the differences was assessed with Student's *t*-test for paired data.

RESULTS

The molecular structure of TBS in the deprotonated colored quinoid form is shown in Fig. 1.

Figure 2 shows the plasma concentration of TBS as a function of time in normal male and female rats; the same data were also plotted on a semi-logarithmic scale.

Table 1 reports the initial plasma disappearance constant rate, the plasma clearance, the initial velocity of uptake and the plasma distribution volume for TBS in male and female rats. While the distribution volume was comparable for the two sexes, the initial plasma disappearance constant rate, plasma clearance and the initial velocity of uptake were higher in females than in males.

Figure 3 shows TBS (10 μM) uptake into sinusoidal liver plasma membrane vesicles followed at the wavelength pair of 576–650 nm ($A_{576-650}$). Similar to what was observed previously [22], the addition of the vesicles was followed by a decrement in absorbance that stabilized in a few seconds. This portion is due mainly to absorption of the dye by the plasma membrane surface, a slight acidification of the medium, light scattering, and equilibration between external and internal vesicular space. This portion of the curve was identical for male and female vesicles, so there was no sex

TABLE 1. Initial plasma disappearance constant rate (*k*), initial velocity of uptake (V), plasma distribution volume (Vd), and plasma clearance (Cl) of tetrabromosulfonephthalein (TBS) in male and female rats

	k (min^{-1})	V (μmol/min/kg)	Vd (mL/kg)	Cl (mL/min/kg)
Males	0.395 ± 0.053	0.593 ± 0.079	37 ± 2	14 ± 2
Females	0.516 ± 0.0068*	0.775 ± 0.102*	39 ± 6	21 ± 3*

Each value is the mean ± SD of four experiments.
* $P < 0.05$.

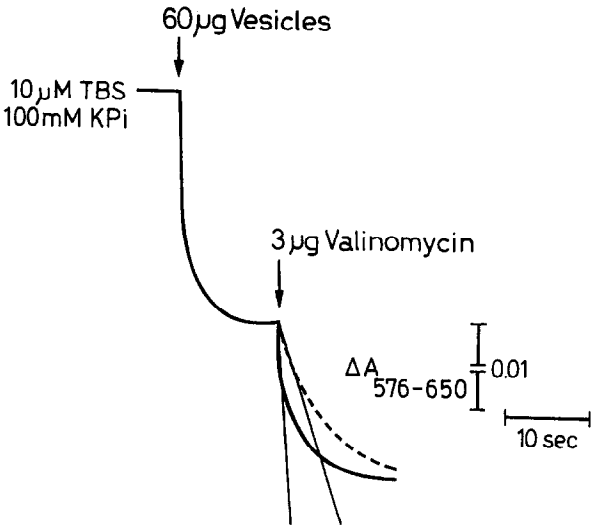

FIG. 3. TBS movements in a liver plasma membrane preparation. Protein (60 μg) diluted 1:1 with 0.1 M HEPES, pH 7.4 (final vol. 25 μL) was added to 1.975 mL of 0.1 M potassium phosphate (KP_i) buffer, pH 8.1, containing 10 μM TBS. Experiments were carried out at room temperature. Valinomycin (3 μg) dissolved in 6 μL methanol was added as shown. Changes in absorbance were followed by a dual wavelength recording spectrophotometer at the wavelength pair 576–650 nm. The initial uptake rate was calculated from the linear portion of the curve in male vesicles (dashed line) and in female vesicles (solid line).

difference in nonspecific binding of the dye and in basal potassium permeability. Creation of a positive-inside membrane potential by the addition of valinomycin in the presence of an inwardly directed potassium gradient was associated with a decrement in absorption, indicating the entry of the dye into the more acidic inner space of the vesicles. The initial uptake rate was calculated from the linear portion of this curve by dividing the amount of the dye transported by protein concentration and by time. As shown in the figure, females presented a higher initial uptake rate of TBS.

Figure 4 shows that the TBS uptake rate in sinusoidal liver plasma membrane vesicles, measured as a function of the dye's concentration, followed a saturable carrier-mediated kinetic for both sexes. As seen in Table 2, V_{max} values were comparable for male and female rats, but K_m values were significantly higher in males than in females.

DISCUSSION

A sex difference has been described in the hepatic handling of organic anions such as bilirubin, BSP, and indocyanine green. These studies have been performed in complex experimental systems such as intact animals, isolated and perfused rat liver, and isolated rat hepatocytes [1, 2, 4, 9]. One hypothesis was that this difference is probably located at the plasma membrane level; therefore, we decided to test it using sinusoidal rat liver plasma membrane vesicles and a convenient organic anion such as TBS.

TBS is an organic anion transported electrogenically in the liver [22]. This dye is a pH indicator phthalein that exists either as a neutral phenolic compound or as a monoanionic quinoid form. This allowed us to study the electrogenic movement of TBS in sinusoidal rat liver plasma membrane vesicles using the spectrophotometric assay described previously.

In the intact animal TBS has pharmacokinetic properties

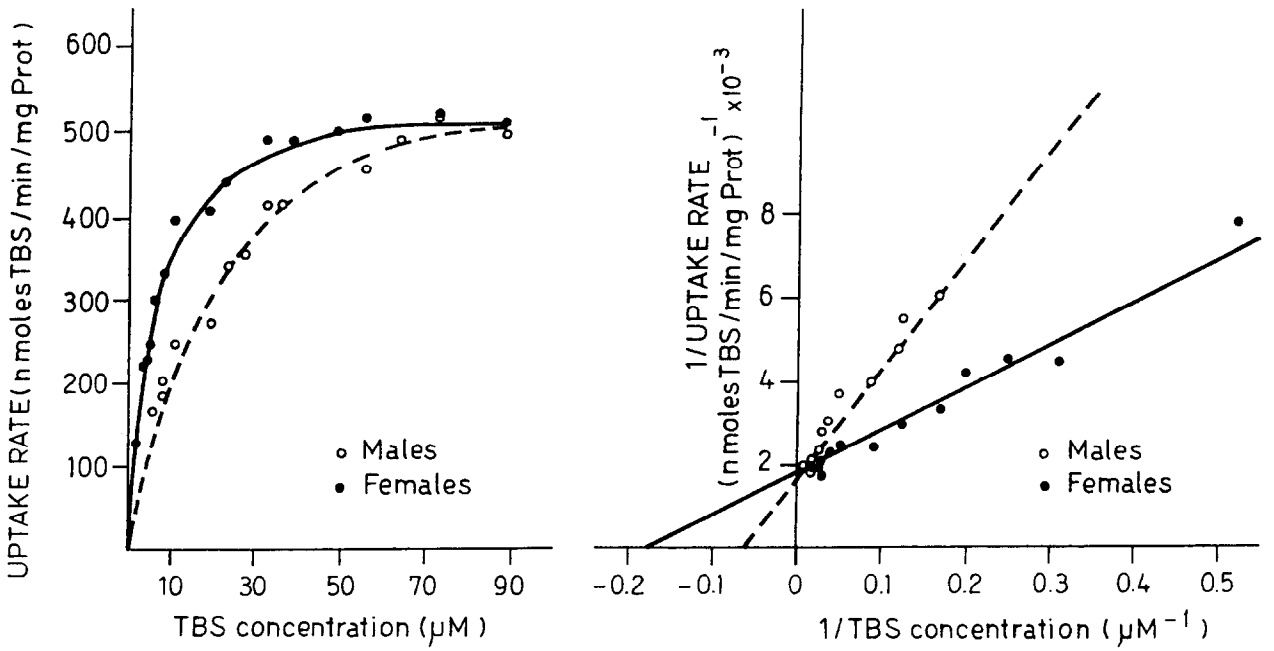

FIG. 4. Kinetic of TBS uptake in sinusoidal liver plasma membrane vesicles from male and female rats. Both curves represent the result of a typical experiment.

TABLE 2. Kinetic parameters for tetrabromosulfonephthalein (TBS) uptake in rat sinusoidal liver plasma membrane vesicles

	V_{max} (nmol/min/mg protein)	K_m (μM)
Males	581 ± 60	17 ± 4.0
Females	544 ± 15	5.5 ± 0.4*

Values are means ± SD of three different vesicle preparations for each group.
* $P < 0.05$.

similar to those of BSP and, like a wide variety of other organic anions including fatty acids, uptake is faster by female than male livers. To investigate further the origin of this sex difference, TBS uptake was measured in sinusoidal rat liver plasma membrane vesicles. The K_m values were higher in males than in females whereas no difference was observed in the V_{max} values. These data strongly suggest that the greater net incorporation of TBS in female hepatocytes is due to a difference in membrane transport rates.

Weisiger and Fitz [38] have pointed out that female hepatocytes have a membrane potential significantly different from that of male hepatocytes. In fact, a less negative membrane potential in females would provide a greater driving force for the electrogenic organic anion transport system.

The present study shows that the sex difference in hepatic uptake of TBS is also located at the sinusoidal hepatic membrane domain and is related to the function of the electrogenic transport system(s).

It remains to be elucidated if there is a sex-related structural difference in the electrogenic transport system(s) or if the greater affinity of the transport system(s) for organic anions may be the result of a more favorable membrane microenvironment. It should be speculated that the reported greater fluidity of the female hepatocyte membrane [39] may indeed enhance the transport system(s) function and be ultimately responsible for the sex difference in organic anion uptake.

As bilitranslocase mediates, at least in part, the electrogenic transport of TBS [22], the role of this protein in the sex difference previously described needs further study.

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